Library Preparation Kit for myBaits®

For targeted DNA sequencing

User Manual

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INTRODUCTION

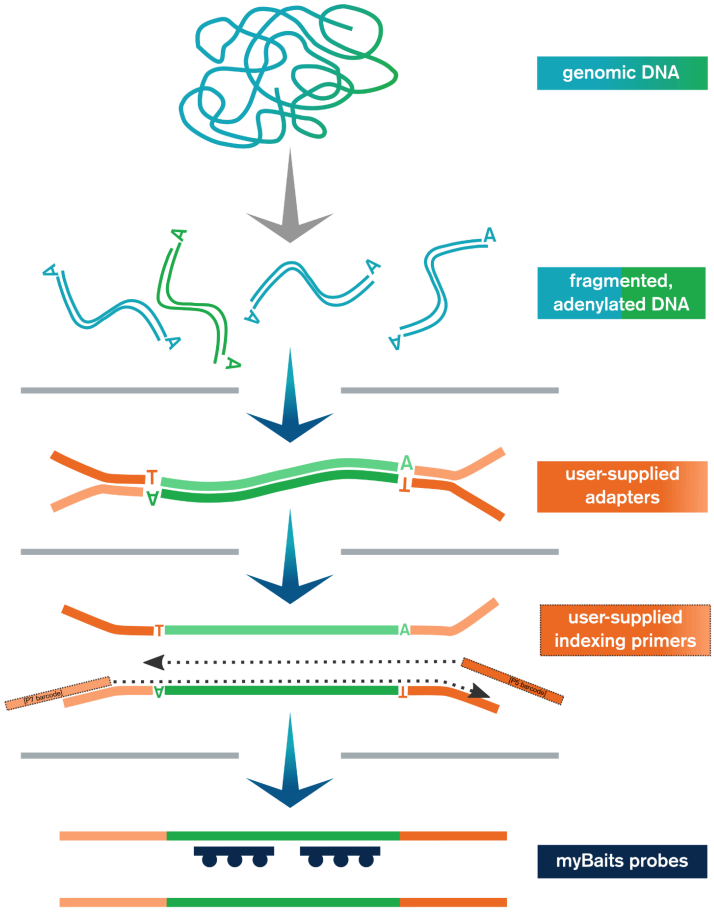
The Library Preparation Kit for myBaits® converts double-stranded DNA to sequencing-ready libraries for the Illumina® platform, prior to enrichment with a myBaits hybridization capture kit. It provides an excellent conversion rate and minimal sequence composition bias.

Input:	1-500 ng dsDNA in water or Tris-based buffer (≤10 mM Tris and ≤0.1mM EDTA)
Shearing:	Enzymatic
Adapter ligation:	A/T-overhang-based
Post-ligation PCR:	Yes
Intended use:	Preparation of NGS libraries from dsDNA, prior to myBaits hybridization capture

Included in the kit are all enzymes necessary for fragmentation, end-repair and adenylation, adapter ligation (with user-supplied adapters), library amplification (with user-supplied indexing primers or kit-supplied P5/P7 primers) both pre- and post-myBaits capture, and SPRI beads for library purification both pre- and post-myBaits capture.

Procedure Overview

1. Genomic or other double-stranded DNA (dsDNA) is enzymatically fragmented, end-polished, and adenylated.
2. User-supplied adapters are ligated to the end-repaired fragments.
3. Ligation products are purified with SPRI beads and then amplified for a limited number of cycles using user-supplied or kit-supplied primers. Libraries are SPRI-purified again and assayed for quantity and morphology.
4. Libraries are optionally pooled and then taken to myBaits capture.



Kit components and stability

Storage Conditions	Reagent	Tubes and volumes			
		32 reaction kit		128 reaction kit	
		Vessels	Volume each	Vessels	Volume each
Box A *Store at -20˚C*	Frag/AT Buffer	1	150 µL	1	590 µL
	Frag/AT Enzyme Mix	1	220 µL	1	890 µL
	Ligation Mix	1	740 µL	2	1480 µL
	Amplification Mix	1	1150 µL	3	1550 µL
	P5/P7 Primer Mix	1	230 µL	1	920 µL
Box B *Store at 4˚C*	SPRI Beads	1	7.5 mL	4	7.5 mL
	Buffer P	1	5.0 mL	2	5.0 mL

The kit is shipped with cold packs. Place boxes in indicated storage temperatures immediately upon arrival.

Reagents required

Reagent	Notes
T-overhang-containing double-stranded adapters	These can be either short ("stubby") adapters OR full-length adapters that contain sample-specific barcodes. If using stubby adapters, indexing primers that add universal P5 and P7 priming sites are also required. See **Appendix A** for further information about adapters and primers.
Molecular grade water	
200 proof ethanol	

Equipment required

Item	Notes
Ice and/or cold block	For reaction setup and benchtop reagent storage during setup
Thermal cycler	
0.2 mL tubes or strips	Compatible with thermal cycler
Plate seals or strip lids	Compatible with selected 0.2 mL tubes or strips
Magnetic particle collector	Compatible with selected 0.2 mL tubes or strips
Assorted nuclease-free tubes	For master mix preparation
Vortexer	
Mini centrifuge	
Digital microfluidic electrophoresis device (dsDNA)	Including consumables. For example, Bioanalyzer® (Agilent)
DNA quantification fluorometer (dsDNA)	Including consumables. For example, Qubit™ (Thermo Fisher)

PROCEDURE

PART 1: Fragmentation and end-repair

1.1 Prepare materials

Reagents	
1-500 ng dsDNA in 40 µL water or buffer containing ≤10 mM Tris and ≤0.1mM EDTA	
Frag/AT Buffer	**KEEP ON ICE**
Frag/AT Enzyme Mix	**KEEP ON ICE**

Equipment
Ice in vessel that can hold reagents and reactions
0.2 mL strips or PCR plate, one well per prep reaction
Lids or film for above compatible with thermal cycler
Thermal cycler

1.2 Program and start the thermal cycler

Final insert length is driven by the time and temperature of the fragmentation reaction. Program and start the thermal cycler according to your fragment length goals, 50 µL volume setting.

Step	Temperature	Time
Lid	105°C	∞
1	4°C	∞
2	30°C	4 or 15 m*
3	65°C	30 m
4	4°C	∞

** See Part 4, section 2 for examples of final amplified libraries using different fragmentation settings. When starting with intact high molecular-weight dsDNA, use 4 minutes for final 500-700 bp average insert lengths, and 15 minutes for final 300-400 bp average insert lengths. For low molecular weight or degraded DNA, optimization will be required, but start with 4 minutes. Samples can vary in their final lengths and sample sets may require experimentation to achieve the desired length distribution. For further assistance, review Appendix B, and contact techsupport@arbor.daicel.com for additional technical support.*

1.3 Set up the fragmentation and end-repair reaction

WORK ON ICE

1. Invert the Frag/AT Buffer and Frag/AT Enzyme Mix 10 times to mix. Briefly centrifuge to collect the material at the bottom of each tube.
2. Build the Frag/AT mastermix. **The following volumes are already adjusted for pipetting error:**

Component	µL / Reaction
Frag/AT Buffer	4.4
Frag/AT Enzyme Mix	6.6
TOTAL	**11**

3. Vortex gently for 4 seconds and briefly centrifuge to collect.
4. Add 10 µL Frag/AT mastermix to each 40 µL DNA sample.
5. Mix by pipetting 10 times using 25 µL volume, or by briefly vortexing. Ensure mixing is consistent between reactions. Centrifuge briefly to collect fluid.
6. Place the reactions in the 4°C thermal cycler and proceed to the next step of the program.

PART 2: Adapter ligation

2.1 Prepare materials

Reagents	Equipment
Adapters at 15 or 3 µM*	Thermal cycler
Ligation Mix **KEEP ON ICE**	Magnetic Particle Collector (MPC)
SPRI Beads **Bring to room temp**	Microcentrifuge
200 proof ethanol (~250 µL per reaction)	Vortexer
Molecular grade water (~90 µL per reaction)	

** For 11-500 ng dsDNA inputs, use 15 µM adapters; for 1-10 ng input, use 3 µM adapters. See Appendix A for additional information on the adapters required for ligation.*

1. Bring the SPRI Beads to room temperature before use.
2. Prepare 80% ethanol solution for washes, ~300 µL per reaction (e.g. 248 µL 200 proof ethanol + 62 µL molecular-grade water, or similar).

2.2 Program the thermal cycler

Step	Temperature	Time
Lid	OFF	∞
1	20°C	15 m

2.3 Set up the ligation reaction

1. Invert the Ligation Mix 10 times to mix.
2. Remove the fragmentation and end-repair reaction tubes from the thermal cycler.
3. Add 5 µL of adapter to each tube.
4. Add 20 µL of Ligation Mix to each tube.
5. Mix by pipetting 10 times using 40 µL volume.
6. Place the reactions in the thermal cycler and start the program, 75 µL volume setting.
7. Prepare a volume of 80% ethanol equal to 300 µL × the number of ligation reactions.
8. Once the incubation ends, remove reactions from the thermal cycler and proceed to 2.4.

2.4 Clean up the ligation reaction

1. Once equilibrated to room temperature, vortex the SPRI Beads to thoroughly resuspend.
2. Add **60 µL** SPRI Beads to each reaction.
3. Mix by pipetting 10 times, or by briefly vortexing. Briefly spin down to collect fluid.
4. Incubate the bead+ligation reaction mixture for 5 minutes at room temperature.
5. Pellet the beads in the MPC for 5 minutes.
6. Remove and discard the supernatant. Leave the reactions in the MPC.
7. Add 150 µL 80% ethanol. Incubate at RT for 30 seconds and remove and discard the ethanol.
8. Repeat step 7 above an additional time for two total washes.
9. Briefly spin down the tubes and return to the MPC.
10. Using a smaller pipette tip than used for the washes, remove any remaining visible ethanol from the bottom of the tube. Remove from the MPC.
11. Let the open vessels dry for 3-5 minutes. Once cracks form in the pellet, they are sufficiently dry.
12. Add 22 µL water and pipette up and down several times to thoroughly resuspend the pellet.

    At this stage, ligation products can be stored for 1 week at -20°C.

PART 3: Library amplification

3.1 Prepare materials

Reagents		Equipment
Ligation reactions from 2.4		Thermal cycler
Primers at 20 µM*		Magnetic Particle Collector (MPC)
Amplification Mix	**KEEP ON ICE**	Microcentrifuge
SPRI Beads	**Bring to room temp**	Vortexer
200 proof ethanol (250 µL per reaction)		
Molecular grade water (65 µL per reaction)		
Buffer P		

** If full-length adapters were used, use kit-supplied P5/P7 Primer Mix. If short adapters were used, use indexing primers at 20 µM. See Appendix A for information on primers for library amplification.*

1. Bring the SPRI Beads to room temperature before use.
2. Prepare 80% ethanol solution for washes, ~310 µL per reaction (e.g. 248 µL 200 proof ethanol + 62 µL molecular-grade water, or similar).

3.2 Program the thermal cycler

Step	Temperature	Time	
1	98°C	45 s	
2	98°C	15 s	×4-10 cycles*
3	60°C	30 s	
4	72°C	45 s	
5	72°C	60 s	
6	12°C	∞	

gDNA input (ng)	cycles for ~1 µg
** 400-500:*	*4*
200-400:	*5*
50-200:	*6*
< 50:	*10-15*

3.3 Set up the amplification reaction

1. (Thaw, if applicable, and) pellet the ligation reactions from step 2.4. Transfer 20 µL of the supernatant to a 0.2 mL reaction well of a strip or plate compatible with your thermal cycler.
2. To each reaction, if full-length adapters with barcodes were used in the ligation reaction, add 5 µL of P5/P7 Primer Mix to each well. If short adapters without barcodes were used in the ligation reaction, add 5 µL of each indexing primer pair (at 20 µM) to each well.
3. Add 25 µL Amplification Mix to each well, and pipette several times to mix. Briefly spin down the tubes.
4. Place the reactions in the thermal cycler and begin the program, 50 µL volume setting.

3.4 Clean up the library amplification reactions

1. Once equilibrated to room temperature, vortex the SPRI Beads to thoroughly resuspend.
2. If starting with high molecular-weight DNA, Add **50 µL** (1× ratio) SPRI Beads to each reaction. If starting with degraded DNA, add **75 µL** (1.5× ratio) SPRI Beads to each reaction.
3. Mix by pipetting 10 times, or by briefly vortexing. Briefly spin down to collect fluid.
4. Incubate the beads+ligation reaction mixture for 5 minutes at room temperature.
5. Pellet the beads in the MPC for 5 minutes.
6. Remove and discard the supernatant. Leave the reactions in the MPC.
7. Add 150 µL 80% ethanol. Incubate at RT for 30 seconds and remove and discard the ethanol.
8. Repeat step 7 an additional time for two total washes.
9. Briefly spin down the tubes and return to the MPC.
10. Using a smaller pipette than used for the washes, remove any remaining visible ethanol from the bottom of the tube. Remove from the MPC.
11. Let the open vessels dry for 3-5 minutes. Once cracks form in the pellet, they are sufficiently dry.
12. Add 30 µL Buffer P and pipette up and down several times to thoroughly resuspend the pellet.
13. Briefly centrifuge the tubes to collect fluid.
14. Pellet on the MPC for 1 minute; remove and save the supernatant.
15. The amplified libraries are now ready for quantification and visualization.

    Amplified libraries can be stored for up to 12 months at -20°C

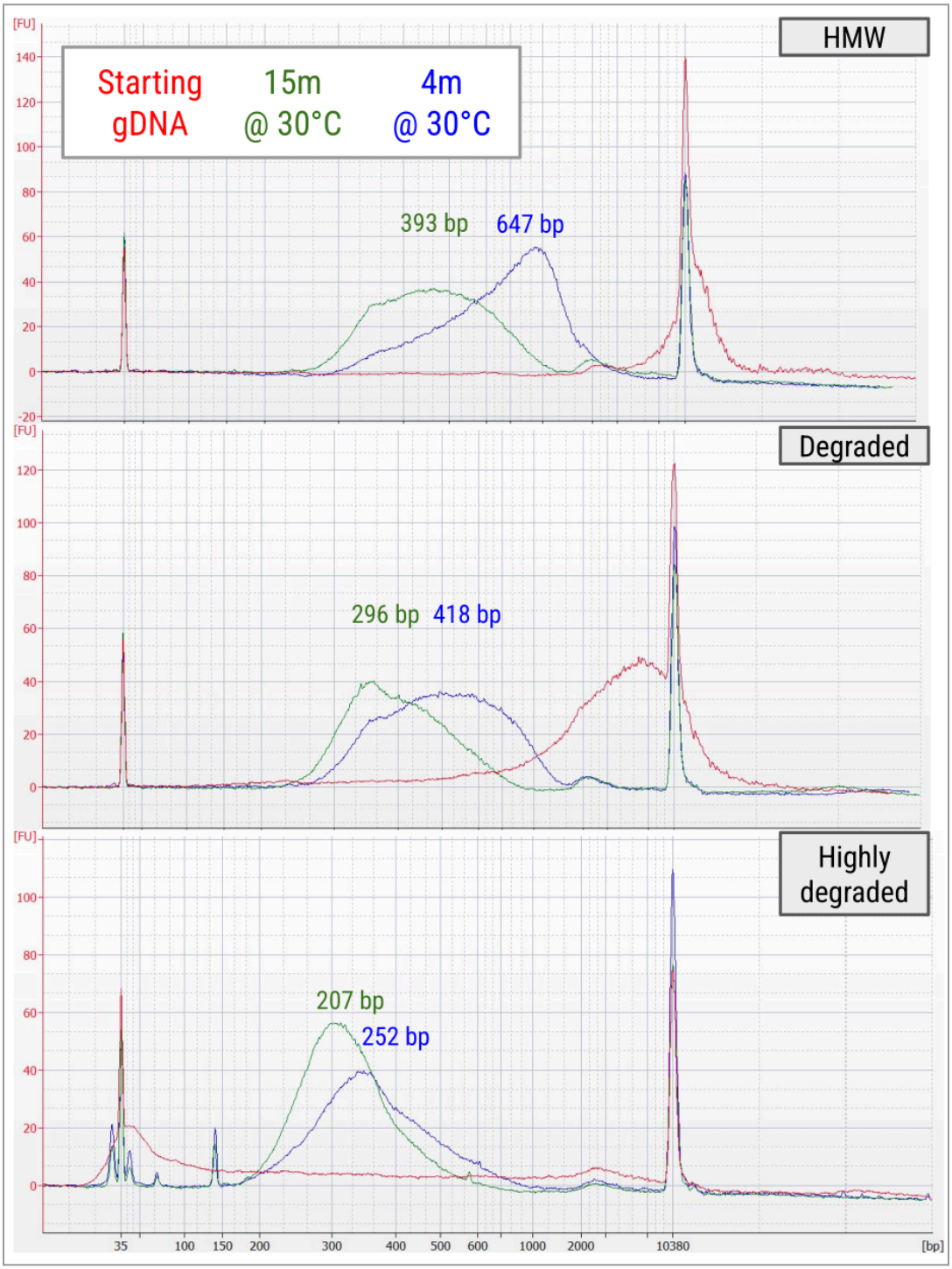
PART 4: Library quantification and visualization

1. Use 2 µL of each library in a fluorescent dsDNA quantification assay, e.g. Qubit High Sensitivity dsDNA Assay.

   If the number of cycles used followed the recommendations in the Library Amplification thermal program, you can expect between 500 ng and 1.5 µg total mass per library. **If your post-amplification yields are not at least triple the input mass of dsDNA** used for input, it is likely the library preparation was suboptimal, and conditions should be reviewed (see Appendix B).

2. Use 1-2 ng of each library in a capillary electrophoresis platform, e.g. Agilent Bioanalyzer.

   Note that after generation of Illumina TruSeq®-style dual 8 bp barcode format libraries, a total of 136 bp of synthetic DNA is added to the starting sample DNA fragments. Libraries should be largely free of adapter dimer (peak at ~120-140 bp), though starting with low amounts of DNA or degraded DNA can result in retention of unused adapters or primers (17-60 nt) or adapter dimer.



Data Figure 1. Example library morphologies following amplification, as visualized on Agilent Bioanalyzer platform. Three different starting genomic DNA samples (top, high-molecular weight [HMW]; middle, degraded; bottom, highly degraded) are shown in red, with post-amplification traces for libraries made from that sample shown in green (using 15 minute fragmentation) and blue (using 4 minute fragmentation). Average insert lengths (total length minus 136 bp adapter) are shown above the primary peak of each library.

PART 5. Preparation for myBaits hybridization capture

To find the appropriate myBaits manual for your kit, visit https://arborbiosci.com/mybaits-manuals/

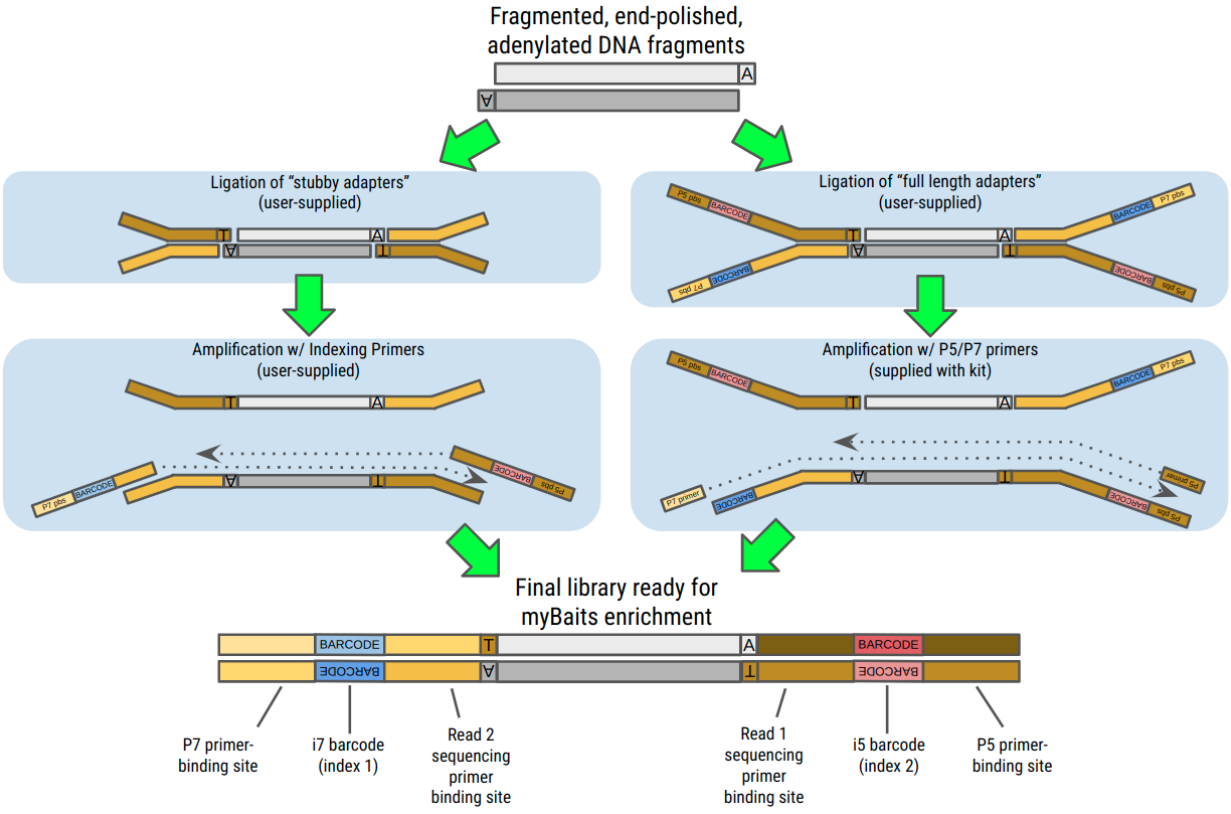
Every myBaits protocol contains specialized guidelines regarding library input mass and, if applicable, number of individual libraries to pool together for capture. These guidelines depend on the type of experiment being performed and the characteristics of the samples, targets, and/or sequencing, so follow the instructions in the appropriate myBaits protocol closely.

Each myBaits target enrichment reaction has space for 7 µL total NGS library volume. Many libraries (or pools of libraries) will require concentration to 7 µL with vacuum centrifugation or other means. Complete this step in advance of your myBaits experiments. Libraries should be suspended in nuclease-free buffer or water.

(*Note*: To generate non-enriched data metrics for libraries prepared with this kit, do not proceed to myBaits hybridization capture, and instead proceed directly to sequencing following Part 4. Non-enriched libraries will need to be sequenced on a separate run as they will have the same barcodes as their enriched counterparts.)

APPENDIX A. Adapters and Primers

The end-repair step of this kit produces 3'-A-tailed dsDNA ends ready for "sticky" ligation to a 5'-T-tailed dsDNA adapter. In Illumina contexts, the adapter is typically either a short configuration that lacks the flowcell binding sites ("P5" and "P7") and sample-specific indexes/barcodes, or a full-length adapter that contains P5, P7, and barcodes. When coupled with short adapters, libraries must be amplified prior to capture with primers that add the P5/P7 sites and barcodes, while with barcoded full-length adapters libraries can be amplified with the universal P5/P7 Primer Mix supplied with the kit. After myBaits capture, both types can be amplified with the P5/P7 Primer Mix.



APPENDIX B. Troubleshooting

My libraries are longer/shorter than desired

Input quality, input purity, fragmentation time, and post-amplification SPRI ratio all interact to determine library insert length. These can be adjusted to tune the desired insert lengths, which for standard myBaits hybridization capture are typically in the 300-500 bp insert length range.

Input quality: The starting length distribution of the dsDNA will often impact the final length distribution (see Data Figure 1).

Input purity: EDTA and carryover from DNA extraction can impact enzymatic activity. Ensure your dsDNA is thoroughly purified and in nuclease-free water or a Tris-based buffer containing ≤0.1 mM EDTA, and is free of viscosity or color.

Fragmentation time: This can be tuned to match your target lengths. We recommend increasing/decreasing in 20% increments to identify the most suitable fragmentation time for your sample set.

SPRI ratio: After amplification, the ratio of SPRI beads will impact the lower length-cutoff, and thus the mean insert length. A 1× SPRI ratio, or 50 µL SPRI beads, during post-amplification cleanup will typically produce lower insert cutoffs like Data Figure 1, "HMW" and "degraded" traces, while a 1.5× SPRI ratio, or 75 µL SPRI beads, will produce lower cutoffs like Data Figure 1, "highly degraded" panel.

My library yields are lower than desired

Yields can vary significantly, but several outcomes can result in a library of sufficient complexity for downstream use. If your yields are less than triple your starting dsDNA input, consider tuning parameters to improve the recovery rate and thereby final library complexity. Input quality, input quantity, amplification cycles, and post-amplification SPRI ratio all interact to determine library yield. Generally speaking, tripling your input will typically ~double the final yield, while you can expect a doubling of yield with every ~2 cycles of indexing amplification. Experimentation will sometimes be required to determine the appropriate pairing of input mass and amplification cycles to achieve your desired yield.

The following are example average yields and insert lengths for libraries prepared from a commercially-sourced HMW human genomic DNA.

	4 min fragmentation		15 min fragmentation	
input (ng)	avg yield (ng)	avg insert length (nt)	avg yield (ng)	avg insert length (nt)
10	112	479	338	292
50	500	502	1194	301
100	1290	444	1730	312
200	1425	465	2726	314

Data Table 1: Example average library yields and insert lengths (final library length minus 136bp) after 4 and 15 minutes of fragmentation time, followed full-length adapter ligation and then 6 amplification cycles, for a range of mass inputs of HMW gDNA.

I have visible “adapter dimer” in my libraries

Adapter dimer, which is often a visible sharp peak around 120-140 bp depending on final adapter configuration, is often dramatically reduced or eliminated altogether via myBaits capture. However if it persists, you can:

- Re-purify the genomic DNA input and elute in Tris-Tween buffer or Buffer E (from myBaits capture kit).
- Use a lower adapter concentration; standard recommendation is 15 µM for 10-500 ng input, 3 µM for 1-10ng, but consider 0.5 µM if adapter dimer remains a problem.
- If full-length adapters were used, try short adapters with indexing amplification instead.
- Reduce post-amplification (either before or after capture) cleanup SPRI ratios from e.g. 1.5 to 1.2×.
- Use an electrophoresis selection system to physically separate the dimers from the rest of the library.