Dovetail GENOMICS

Dovetail™ Hi-C

User Manual
version 1.4

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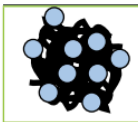
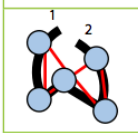
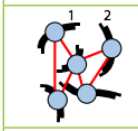
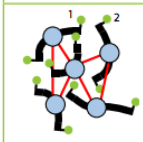
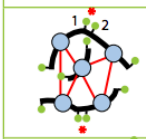
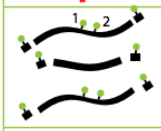
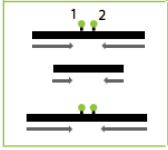
General Information

Overview of Dovetail™ Hi-C Proximity Ligation Method

	A	Dovetail™ Hi-C libraries start with endogenous chromatin.
	B	Crosslinking (red lines) the chromatin creates a stabilized nucleosome (blue circles) scaffold.
	C	Restriction endonucleases digest the cross-linked chromatin.
	D	Biotin (green dots) mark digested DNA ends (black lines).
	E	Proximity ligation creates chimeric molecules (ex. 1 and 2).
	F	The crosslinks are reversed.
	G	DNA is purified, and enriched for ligation-containing chimeric molecules. Libraries sequenced as paired-end short reads

Sequencing

Dovetail™ Hi-C Kit in combination with Dovetail™ Library Module for Illumina® and Dovetail™ Primer Set for Illumina® are designed to create Illumina®-compatible Hi-C sequencing libraries. The proximity-ligated DNA prepared using Dovetail™ Hi-C Kit can also be used with a range of commercially available library preparation kits. The Hi-C libraries should be sequenced as paired-end short reads; the sequencing depth varies depending on the genome size and intended application.

Library Quality Control Analysis

Dovetail's QC analysis allows users to validate the quality of the Hi-C library before deep sequencing. Dovetail provides all kit users with free access to the QC analysis. The QC process requires an input assembly and 1-2 million 2 x 75 or longer paired-end reads sequenced from the Hi-C library.

Data Analysis

Genome Scaffolding: using Dovetail HiRise™ scaffolding pipeline or open source scaffolding tools.

Please refer to the table below for sequencing guidelines if using Dovetail HiRise™. When more than one Hi-C library is required, the number of read pairs indicated in the table is generated from the pooled Hi-C libraries. For example, for a 2 Gb complex genome we recommend preparing 2 Hi-C libraries, pooling them, then sequencing at least 250 million read pairs from the combined pool.

	Simple Genomes		**Complex Genomes**	
Genome size (Gb)	No. Hi-C libraries	No. Read pairs to sequence (Million)	No. Hi-C libraries	No. Read pairs to sequence (Million)
1	1	100	1	100
2	1	200	2	250
3	1	300	2	450
4	2	400	3	500
5	2	500	3	850

"**Simple Genomes**" are diploid or haploid, have repetitive content of less than 30% and heterozygosity of less than 0.005%. Examples of simple genomes include humans, many mammals and some fish and birds.

"**Complex Genomes**" contain any of the following: polyploidy, repeat content above 30%, heterozygosity above 0.005%. If any of the above metrics are unknown, use the complex genome sequencing guidelines. Examples of complex genomes include many plants, salmonid fish, and amphibians.

Structural Variation Detection: using Dovetail Selva™ analysis pipeline or publicly available tools. Selva™ analysis requires 150 - 300 M paired-end (2 x 150 bp) reads as input data.

3D Genome conformation, genome organization (TADs, A/B compartments, chromatin loops): using publicly available tools.

Dovetail™ Hi-C Kit Components & Storage

Each kit contains a sufficient supply of materials to perform 8 reactions. Dovetail™ Hi-C Kit comes as two boxes. Store the boxes as listed below immediately upon receipt.

Dovetail™ Hi-C Kit Box 1
(PN DG-HiC-002)

Components	Cap Color	Storage
TE Buffer pH 8.0	None	2°C to 8°C
Wash Buffer	white label	
TWB Solution		
2X NTB Solution		
LWB Solution		
NWB Solution		
Chromatin Capture Beads		
Crosslink Reversal Buffer		
Streptavidin Beads		
10X RBC Lysis Buffer		
20% SDS		
40mM Calcium Chloride		

Dovetail™ Hi-C Kit Box 2
(PN DG-HiC-004)

Components	Cap Color	Storage
Restriction Digest Buffer		-30°C to -10°C
Restriction Digest Enzyme Mix		
End Fill-in Buffer		
End Fill-in Enzyme Mix		
Intra-Aggregate Ligation Buffer		
Intra-Aggregate Ligation Enzyme Mix		
Proteinase K		
Collagenase		
HotStart PCR Ready Mix		
250 mM DTT		

Optional Add-on Modules: Components & Storage

Dovetail™ Hi-C Kit Filter Set (PN DG-HiC-005)

Components	Cap Color	Storage
50 µm Filters		**Room Temp**
100 µm Filters		

Dovetail™ Primer Set For Illumina® (PN DG-PRS-001)

Components	Cap Color	Storage
Index Primers (x8, different)		**Store at -30°C to -10°C**
Universal PCR Primer		

Dovetail™ Library Module For Illumina® (PN DG-LIB-001)

Components	Cap Color	Storage
End Repair Enzyme Buffer		**Store at -30°C to -10°C**
End Repair Enzyme Mix		
Ligation Enhancer		
Ligation Enzyme Mix		
Adaptor for Illumina		
USER Enzyme Mix		

User Supplied Reagents, Consumables & Equipment

Reagents		
SPRIselect™ beads	Beckman Coulter	B23317
37% Formaldehyde solution	Sigma-Aldrich	F8775
1X PBS, pH 7.4	Thermo Fisher Scientific	10010023
80% EtOH	Generic	-
DNase / RNase- free distilled water	Invitrogen	10977015

Consumables and Equipment	
1.5 mL Low binding microcentrifuge tubes	Generic Supplier
0.2 mL PCR tubes	
5 mL centrifuge tubes (for plant samples)	
15 mL centrifuge tubes (for blood samples)	
Pipets and pipet tips	
1 mL wide bore pipet tips (for plant samples)	
Magnetic separation rack for 0.2 mL and 1.5 mL tubes	
Agitating thermal mixer	
Thermal cycler	
Vortex	
Centrifuge	
Dry ice/petri dish/razor or liquid nitrogen/mortar and pestle (for animal tissue and plant samples)	
Hemocytometer (for cell culture and blood samples)	
1 mL BD Sterile Tuberculin Syringes (for plant and insect samples)	Becton Dickinson 309659
Qubit® Fluorometer	Thermo Fisher Scientific
Qubit™ dsDNA HS Assay Kit	Thermo Fisher Scientific Q32854
Qubit™ Assay Tubes	Thermo Fisher Scientific Q32856
TapeStation System or Bioanalyzer	Agilent
Bioruptor® Pico sonicator	Diagenode
Pippin Prep™ (Optional)	Sage Science

Supported Sample Types

There are six supported sample types, each with a distinct protocol for sample preparation and crosslinking (Stage 1). **We recommend using one sample type at a time.**

Supported Sample Type	Protocol to Follow for Sample Preparation & Crosslinking (Stage 1)
Mammalian tissues (muscle, brain, heart, spleen)	Stage 1A, page 13
Mammalian cell culture	Stage 1B, page 14
Mammalian (non-nucleated) blood	Stage 1C, page 15
Non-mammalian (nucleated) blood (fish, bird and reptile blood)	Stage 1D, page 16
Young plant leaves	Stage 1E, pages 17 - 19
Starved mature insects	Stage 1F, page 21

If your desired sample type is not listed in the table above, please contact our technical support team at support@dovetail-genomics.com or +1 (831) 233-3780.

Sample Guidelines

Tissue

Tissue samples with high cellularity and low-fat content are ideal. Recommended tissues are listed below, ordered from most preferred to least preferred:

1. Blood
2. Sperm Cells
3. Spleen
4. Heart
5. Muscle

The kit does not support fat, bone or similar tissue types. Liver tissue is not recommended.

Tissue samples should be taken from a live or very recently deceased specimen and snap-frozen in liquid nitrogen as soon as possible after harvest. Store samples at -80°C until use. Avoid freeze-thaw cycles of tissue samples. Do not use samples which have been freeze-dried. The kit has not been validated with samples preserved in RNAlater™ or ethanol.

Cells

Fresh cells yield better results. Avoid freeze thaw cycles.

Adherent cells can be disassociated using trypsin. Wash the cells with at least 5 mL 1X PBS to remove trypsin before starting the Hi-C protocol.

Blood

Blood samples must have an anticoagulant added. EDTA is the anticoagulant of choice. Blood collection tubes pre-coated with EDTA can also be used. Blood samples collected with heparin and ACD-A were also successfully tested.

Fresh blood samples will perform better. Fresh samples should be stored at 4°C and used within three days of collection. Otherwise, flash freeze blood samples in liquid nitrogen and keep at -80°C until use.

Plants

We recommend the following plant tissues, ordered from most preferred to least preferred:

1. Leaves of plants at the one or two-leaf seedling stage, or cotyledons for species that are abundant in polyphenolics and/or polysaccharides, such as cotton or rose
2. Very young leaves from more mature plants
3. Leaves collected from plants that are pretreated in the dark for 2-3 days
4. Tissue from young plants other than leaves

Flash freeze harvested leaves immediately in liquid nitrogen and store at -80°C until use.

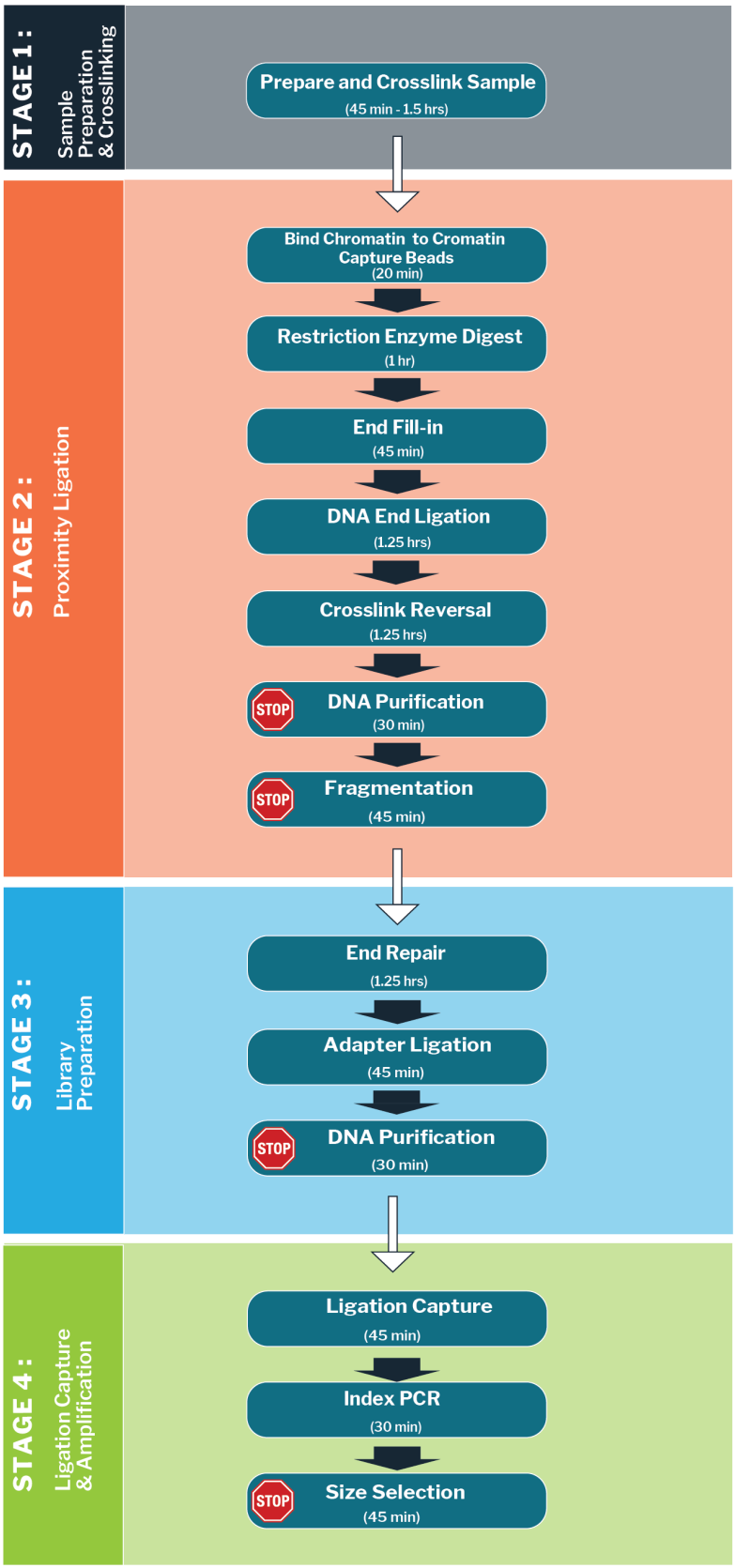
Insects

A major concern for preparing Hi-C libraries from insects is potential contamination from their food. Therefore, the recommended developmental stages of arthropods in the preferred order are:

1. Embryos
2. Newly hatched larvae
3. Early pupae
4. Adults

We recommend degutting adults prior to freezing. Alternatively, they may be starved for a few days prior to freezing. Flash freeze the individuals in liquid nitrogen, either in bulk for inbred species or individually for outbred species, and store at -80°C prior to use.

Dovetail™ Hi-C Protocol Overview



Stage 1. Sample Preparation & Crosslinking

Getting Started

1. Kit-supplied reagents are listed in **bold blue**, user-supplied reagents are listed in **bold black**.
2. Use good laboratory practices, including thawing, vortexing and quick spinning reagents before use.
3. Agitating thermal mixer should be set at 1250 rpm for 1.5 mL tubes.
4. When placing the sample on the magnet, always wait until the solution looks clear and the beads have fully separated before removing the supernatant.

Stage 1A: Crosslinking Human and Animal Tissue Samples (Box 1 & 2)

- Make sure the PBS and formaldehyde added to the sample are freshly mixed.
- Incubate 20% SDS at 37°C for 15 min before use.

NOTE: Keep tissue sample frozen until it is in the tube containing the formaldehyde solution.

1. Weigh out 20-40 mg of frozen tissue sample.
2. Disrupt the tissue either by coarsely chopping it with a razor blade on a petri dish placed on dry ice or by grinding it with a mortar and pestle in liquid nitrogen.
3. Transfer the disrupted tissue sample to a 1.5 mL microcentrifuge tube which contains:

1 mL	**1X PBS**
40.5 µl	**37% Formaldehyde**

NOTE: All the steps after this point are performed at room temperature.

4. Vortex tube for 30s to mix.
5. Rotate tube at room temperature for 20 min. The tissue sample should not settle.
6. Spin tube for 1 min at 15,000 x *g*. Pipet off and discard supernatant.
7. Wash the pellet with 300 µl **Wash Buffer** by pipetting up and down 10 times to resuspend, spinning the tube at 15,000 x *g* for 1 min and removing the supernatant.
8. Repeat step 7 once, for a total of 2 washes.
9. After the second wash, add to pellet:

99 µl	**Wash Buffer**
1 µl	**40 mM Calcium Chloride**
25 µl	**1 mg/mL Collagenase**

10. Incubate for 1 hr at 37°C, in an agitating thermal mixer (1250 rpm).
11. After incubation, add 6.3 µl **20% SDS** to the sample
12. Pipet up and down to break up any large clumps. Briefly vortex to resuspend.
13. Spin the tube at 15,000 x *g* for 1 min.
14. **Transfer the SUPERNATANT to a new 1.5 mL microcentrifuge tube.**
15. Proceed to Proximity Ligation Protocol.

Stage 1B: Crosslinking Cell Culture Samples (Box 1)

- Make sure the PBS and formaldehyde added to the sample are freshly mixed.
- Incubate 20% SDS at 37°C for 15 min before use.

NOTE: All the steps for this sample type are performed at room temperature.

1. Pellet 0.5 x 10^6 cells for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
2. Add to pellet:
   1 mL **1X PBS**
   40.5 µl **37% Formaldehyde**
3. Pipet up and down 10 times to resuspend.
4. Rotate tube at room temperature for 20 min. Cells should not settle.
5. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
6. Add to pellet 1 mL **Wash Buffer** and pipet up and down 10 times to resuspend.
7. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
8. Repeat steps 6 and 7 once, for a total of 2 washes.
9. Add to pellet:
   50 µl **Wash Buffer**
   2.5µl **20% SDS**
10. Pipet up and down to break up any large clumps. Briefly vortex to resuspend.
11. Proceed to Proximity Ligation Protocol.

Stage 1C: Crosslinking Mammalian (Non-Nucleated) Blood Samples (Box 1)

Samples should be normalized to 0.5 x 10^6 white blood cells (step 5 below). This will generally require 300 µl – 1000 µl of blood. If less input is used, the yield will decrease. The kit supplies enough RBC Lysis Buffer to lyse 11 mL of blood or 1.25 mL of blood per reaction if eight blood samples are prepared.

- Make sure the PBS and formaldehyde added to the sample are freshly mixed.
- Incubate 20% SDS at 37°C for 15 min before use.
- Prepare 1X RBC Lysis Buffer by diluting the kit-supplied 10X RBC Lysis Buffer in DNase and RNase- free distilled water.

NOTE: All the steps for this sample type are performed at room temperature.

1. Place in 15 mL tube:
   1 volume Fresh or thawed mammalian blood sample
   10 volumes **1X RBC Lysis Buffer**
2. Pipet mix the sample and incubate at room temperature for 5 min.
3. Pellet for 5 min at 500 x *g*. Pipet off and discard supernatant.
   Use a 100 µl pipet tip to remove any residual supernatant.
4. Resuspend pellet in 1ml **1X PBS**. Carefully remove any cell clumps that do not break apart with pipetting.
5. Count the cells in your resuspended sample and transfer 0.5 x 10^6 cell aliquot into a fresh 1.5 mL tube.
6. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
7. Add to pellet:
   1 mL **1X PBS**
   40.5 µl **37% Formaldehyde**
8. Pipet up and down 10 times to resuspend.
9. Rotate tube at room temperature for 20 min. Cells should not settle.
10. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
11. Add to pellet 1 mL **Wash Buffer** and pipet up and down 10 times to resuspend.
12. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
13. Repeat steps 11 and 12 once, for a total of 2 washes.
14. Add to pellet:
    50 µl **Wash Buffer**
    2.5 µl **20% SDS**
15. Pipet up and down to break up any clumps. Briefly vortex to resuspend.
16. Proceed to Proximity Ligation Protocol.

Stage 1D: Crosslinking Non-Mammalian (Nucleated) Blood Samples (Box 1)

- Make sure the PBS and formaldehyde added to the sample are freshly mixed.
- Incubate 20% SDS at 37°C for 15 min before use.

NOTE: All the steps for this sample type are performed at room temperature, except for step 2, if using frozen blood sample.

1. Prepare a 1.5 mL tube with 400 µl of **1X PBS**.
2. Thaw blood sample rapidly at 37°C, if using frozen blood sample.
3. Vortex the blood sample briefly then transfer 5 µl of sample to the 1.5 mL tube with PBS.
4. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
5. Add to pellet:
   1 mL **1X PBS**
   40.5 µl **37% Formaldehyde**
6. Pipet up and down 10 times to resuspend.
7. Rotate tube at room temperature for 20 min. Cells should not settle.
8. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
9. Add to pellet 1 mL **Wash Buffer** and pipet up and down 10 times to resuspend.
10. Pellet for 5 min at 2,500 x *g*. Pipet off and discard supernatant.
11. Repeat steps 9 and 10 once, for a total of 2 washes.
12. Add to pellet:
    50 µl **Wash Buffer**
    2.5µl **20% SDS**
13. Pipet up and down to break up any clumps. Briefly vortex to resuspend.
14. Proceed to Proximity Ligation Protocol.

Stage 1E: Crosslinking Plant Tissue Samples (Box 1 & Dovetail™ Hi-C Filter Set).

NOTE: There are two methods for preparing and grinding plant samples. Option A (recommended option) uses Becton Dickinson Medimachine System for grinding and Option B uses a mortar and pestle, if a Medimachine is not available.

Option A.

- Incubate the kit-supplied 20% SDS at 37°C for 15 min before use.

1. Place 250 mg of plant tissue in a small mortar sitting in liquid nitrogen.
2. Grind leaves to a coarse powder. Keep the leaves frozen while grinding.
3. Transfer the ground sample to a 5 mL tube which contains:
   2 mL **1X PBS**
   81 µl **37% Formaldehyde**
4. Vortex sample for 30s to mix.
5. Rotate tube at room temperature for 15 min. Tissue should not settle.
6. Spin tube for 5 min at 5000 x *g* at room temperature. Carefully remove supernatant. Do not disturb the pellet.
7. Resuspend pellet in 2 mL **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
8. Pellet for 5 min at 5000 x g at 4°C. Repeat the centrifugation if debris remain floating. Carefully remove supernatant.
9. Resuspend pellet in 1 mL **Wash Buffer**. Keep tube on ice.
10. Prewet the Medicon by pipetting 1 mL **Wash Buffer** onto both sides of the blade. Rotate the blade to ensure wetting of the entire filter. Remove the buffer with a 1 mL syringe.
11. Using a wide bore 1ml pipet tip, transfer your 1 mL resuspended sample from the 5 mL tube onto the Medicon. Insert the Medicon into the BD Medimachine. Retain the 5 mL tube on ice, you will use it during the second round.

First Grind:

12. Grind sample for 60 sec. Pipet mix to redistribute sample in the Medicon. Grind sample again for 60 sec.
13. Once the tissue is disaggregated, remove the Medicon from the BD Medimachine. Insert a 1 mL syringe in the syringe port to recover the suspension.
14. Gently filter the suspension through a **50 µm filter** into a new 5 mL tube. Store the tube on ice.

Second round:

15. Rinse the 5 mL tube which you retained at stage 11 with 1 mL **Wash Buffer** to recover any remaining tissue/debris and pipet onto the Medicon.
16. Grind sample for 60 sec. Pipet mix to redistribute sample in the Medicon. Grind sample again for 60 sec.
17. Remove the Medicon from the BD Medimachine. Insert a 1 mL syringe in the syringe port to recover the ground sample.

18. Gently filter the ground sample through the same **50 µm filter** into the same 5 mL tube of step 14. This tube should now contain a total volume of ~2 mL of filtered sample. Keep the tube stored on ice.

Third round:

19. Add 1 mL **Wash Buffer** onto the Medicon for a thorough rinsing.
20. Grind for 60 sec. Pipet mix to redistribute sample in the Medicon. Grind again for 60 sec.
21. Remove the Medicon from the BD Medimachine. Insert a 1 mL syringe in the syringe port to recover the ground sample.
22. Gently filter the ground sample through the same **50 µm filter** into the same 5 mL tube of step 14. This tube should now contain a total volume of ~3 mL of filtered sample.
23. Pellet at 2500 x g for 5 min at room temperature. Remove supernatant.
24. Resuspend pellet in 1 mL **Wash Buffer.** Vortex briefly until the pellet is fully resuspended.
25. Transfer sample to a 1.5 mL tube.
26. Pellet at 2500 x *g* for 5 min at room temperature. Remove supernatant.
27. Wash pellet with 1 mL **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
28. Pellet for 5 min at 2500 x *g*. Remove supernatant.
29. Resuspend pellet in 100 µl **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
30. Add 5 µl of **20% SDS**.
31. Pipet up and down to break up clumps. Briefly vortex to mix
32. Incubate at 37°C for 15 minutes, in an agitating thermal mixer (1250 rpm).
33. Proceed to Proximity Ligation Protocol.

Option B.

- If not already frozen, snap-freeze freshly collected young leaves immediately in liquid nitrogen before beginning step 1.
- Ensure the PBS and formaldehyde added to the sample are freshly mixed
- Incubate the kit-supplied 20% SDS at 37°C for 15 min before use.

1. Place 250 mg of plant tissue in a small mortar sitting in liquid nitrogen.
2. Grind leaves for 10-20 minutes until they have the consistency of flour (see figure below of desired consistency). Keep the leaves frozen while grinding.

NOTE: All the steps after this point are performed at room temperature.

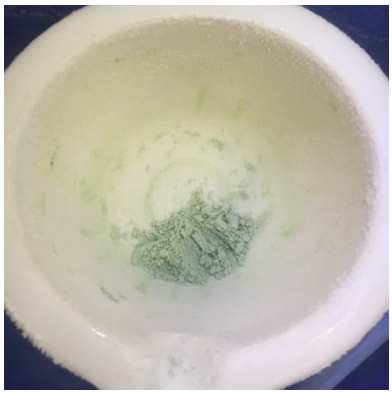
3. Transfer the ground sample to a 5 mL tube which contains:
   2 mL **1X PBS**
   81 µl **37% Formaldehyde**
4. Vortex sample for 30s to mix.
5. Rotate tube at room temperature for 15 min. Tissue should not settle.
6. Spin tube for 5 min at 5000 x *g*. Carefully remove supernatant. Do not disturb the pellet.
7. Resuspend pellet in 2 mL **Wash Buffer**. Vortex briefly to fully resuspend the pellet.

8. Using an attachable 1 mL syringe, gently push the 2 mL resuspended sample through a **200 µm filter** into a new 5 mL tube.
9. If the filter clogs, replace with a new **200 µm filter** and continue until all the sample has been filtered.
10. Gently pass an additional 1 mL **Wash Buffer** though the **200 µm filter** into the 5 mL tube. Your tube should now contain a total volume of ~3 mL.
11. Using the same syringe but changing the filter to a **50 µm** one, filter the 3 mL sample into a new 5 mL tube.
12. Pellet for 5 min at 2500 x *g*. Remove supernatant.
13. Resuspend pellet in 1 mL **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
14. Transfer sample to a 1.5 mL tube.
15. Pellet for 5 min at 2500 x *g*. Remove supernatant.
16. Wash pellet with 1 mL **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
17. Pellet for 5 min at 2500 x *g*. Remove supernatant.
18. Resuspend pellet in 100 µl **Wash Buffer**. Vortex briefly to fully resuspend the pellet.
19. Add 5 µl of **20% SDS**.
20. Pipet up and down to break up any clumps. Briefly vortex to resuspend.
21. Incubate at 37°C for 15 minutes, in an agitating thermal mixer (1250 rpm).
22. Proceed to Proximity Ligation Protocol.

Examples of insufficient (A) and sufficient (B) grinding.

A

B



Stage 1F: Crosslinking Insect Samples (Box 1 & Dovetail™ Hi-C Filter Set)

- Ensure the insect body excludes the abdomen unless the sample has been starved. Gut contents may cause DNA contamination of the library.
- Make sure the PBS and formaldehyde added to each sample are freshly mixed
- Incubate the kit-supplied 20% SDS at 37°C for 15 min before use.
- Use 1 ml wide bore pipet tips.

1. Place up to 100 mg of insect tissue in a small mortar sitting in liquid nitrogen.
2. Grind sample to a fine powder. Keep the tissue frozen while grinding.

NOTE: All the steps after this point are performed at room temperature.

3. Transfer the ground sample to a 1.5 mL tube which contains:
   1 mL **1X PBS**
   40.5 µl **37% Formaldehyde**
4. Vortex sample for 30s to mix.
5. Rotate tube at room temperature for 20 min. Tissue should not settle.
6. Pellet for 5 min at 2,500 x *g*. remove supernatant.
7. Add 1 mL **Wash Buffer**, vortex briefly until the pellet is fully resuspended.
8. Using an attachable 1 mL syringe, gently push the 1 mL resuspended sample through a **200 µm filter** into a clean 5 mL tube.
9. If the filter clogs, replace with a new **200 µm filter** and continue until all the sample has been filtered
10. Gently pass an additional 1 mL **Wash Buffer** though the **200 µm filter** into the 5 mL tube. Your tube should now contain a total volume of 2 mL.
11. Using the same syringe but changing the filter to a **50 µm** one, filter the 2 mL sample into a new 5 mL tube.
12. Pellet for 5 min at 2500 x *g*. Remove supernatant.
13. Resuspend pellet in 1 mL **Wash Buffer.** Vortex briefly until the pellet is fully resuspended.
14. Transfer sample to a 1.5 mL tube.
15. Pellet for 5 min at 2500 x *g*. Remove supernatant.
16. Wash pellet with 1 mL **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
17. Pellet for 5 min at 2500 x *g*. Remove supernatant.
18. Resuspend pellet in 100 µl **Wash Buffer**. Vortex briefly until the pellet is fully resuspended.
19. Add 5 µl of **20% SDS**.
20. Pipet up and down to break up clumps. Briefly vortex to resuspend.
21. Incubate at 37°C for 15 minutes, in an agitating thermal mixer (1250 rpm).
22. Proceed to Proximity Ligation Protocol.

Stage 2. Proximity Ligation

I. Bind Chromatin to Chromatin Capture Beads (Box 1)

1. Allow **Chromatin Capture Beads** to reach room temperature. Vortex to resuspend.
2. Add 100 µl **Chromatin Capture Beads** to the sample tube.
3. Pipet up and down 10 times. Make sure the beads are fully resuspended.
4. Incubate for 10 min at room temperature off magnet.
5. Quick spin the tube and place on the magnet for 5 min. Remove supernatant. Use caution as the bead pellet may be loose.
6. Remove the tube from the magnetic rack, wash beads with 200 µl **Wash Buffer**: pipet up and down 10 times to resuspend the beads then place tube on the magnet for 1 min. Remove supernatant.
7. Repeat step 6 for a second wash.

II. Restriction Enzyme Digest (Box 2)

1. After the second wash has been removed, remove the tube from the magnetic rack then add to beads:
   50 µl **Restriction Digest Buffer**
   1 µl **Restriction Digest Enzyme Mix**
2. Pipet up and down 10 times. Make sure the beads are fully resuspended.
3. Incubate for 1 hr at 37°C, in an agitating thermal mixer (1250 rpm).

III. End Fill-In (Box 1 & 2)

1. Quick spin the tube and place on the magnet for 1 min. Remove supernatant.
2. Remove the tube from the magnetic rack, wash beads with 200 µl **Wash Buffer**: pipet up and down 10 times to resuspend the beads then place tube on the magnet for 1 min. Remove supernatant.
3. Repeat step 2 for a second wash.
4. Remove the tube from the magnetic rack then add to beads:
   50 µl **End Fill-in Buffer**
   1 µl **End Fill-in Enzyme Mix**
5. Pipet up and down 10 times. Make sure the beads are fully resuspended.
6. Incubate for 30 min at 25°C, in an agitating thermal mixer (1250 rpm).

IV. Intra-Aggregate DNA End Ligation (Box 1 & 2)

1. Quick spin the tube and place on the magnet for 1 min. Remove supernatant.
2. Remove the tube from the magnetic rack, wash beads with 200 µl **Wash Buffer**: pipet up and down 10 times to resuspend the beads then place tube on the magnet for 1 min. Remove supernatant.

3. Repeat step 2 for a second wash.
4. Remove the tube from the magnetic rack then add to beads:
   250 µl **Intra-Aggregate Ligation Buffer**
   1 µl **Intra-Aggregate Ligation Enzyme Mix**
5. Pipet up and down 10 times. Make sure the beads are fully resuspended.
6. Incubate for 1 hr at 16°C in an agitating thermal mixer (1250 rpm). For convenience, this ligation reaction can proceed overnight at 16°C, in an agitating thermal mixer (1250 rpm).

V. Crosslink Reversal (Box 1 & 2)

NOTE: Incubate Crosslink Reversal Buffer at least 10 min at 37°C before use to dissolve precipitates.

1. Quick spin the tube and place on the magnet for 1 min. Remove supernatant.
2. Remove the tube from the magnetic rack then add to beads:
   50 µl **Crosslink Reversal Buffer**
   1 µl **Proteinase K**
3. Pipet up and down 10 times. Make sure the beads are fully resuspended.
4. Incubate for 15 min at 55°C, followed by 45 min at 68°C, in an agitating thermal mixer (1250 rpm).
5. Quick spin the tube and place on the magnet for 1 min.
6. **Transfer 50 µl of the SUPERNATANT to a new 1.5 mL tube.** Discard beads.

VI. DNA Purification on SPRIselect™ Beads (Box 1)

NOTE: 80% EtOH should be freshly prepared.

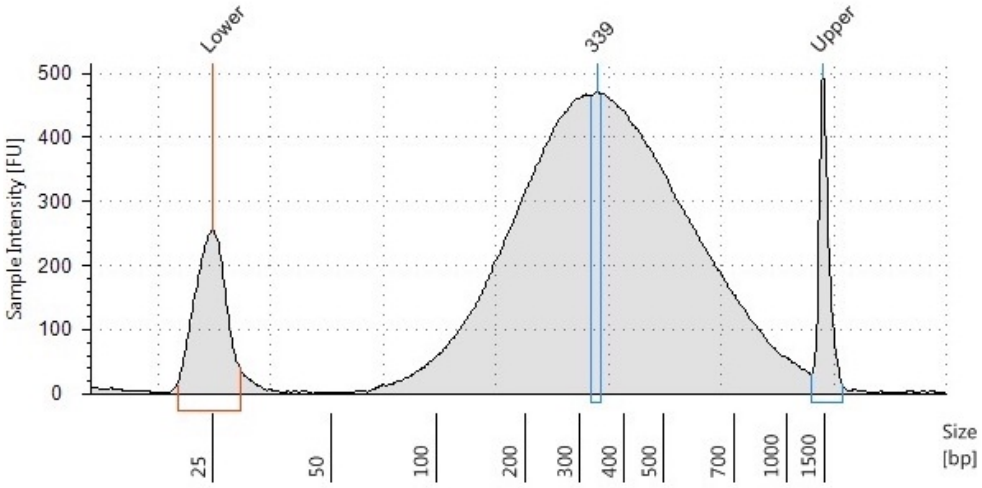
1. Vortex **SPRIselect™ Beads** for 30 sec to resuspend.
2. Add 100 µl of resuspended **SPRIselect™ Beads** to the 1.5 mL sample tube.
3. Vortex to resuspend, quick spin and incubate for 5 min off magnet.
4. Quick spin the tube and place on the magnet for 5 min. Remove supernatant.
5. Leave tube on the magnet, and wash beads twice with 250 µl **80% EtOH**. Do not resuspend the beads for these washes. Simply add the EtOH, wait for 1 min then remove the EtOH wash.
6. After the second wash, quick spin the tube and place on the magnet for 1 min. Use a pipet with a fine tip to remove the last EtOH traces.
7. Air dry beads for 5 min on the magnet until no residual EtOH remains. Do not over dry.
8. Off the magnet, resuspend beads in 54 µl **TE Buffer pH 8.0**.
9. Vortex briefly, quick spin and incubate for 5 min at room temperature off magnet.
10. Quick spin the tube and place on the magnet for 1 min.
11. **Transfer 52 µl of the SUPERNATANT to a new tube.** Discard beads.
12. Quantify the sample using a Qubit Fluorometer and Qubit dsDNA HS Kit. For each sample you should recover more than 200 ng DNA. If less than 100 ng of DNA is recovered do not continue with the sample.

The purified DNA sample can be stored at -20°C for up to 6 months.

VII. Fragmentation (Box 1)

NOTE: See Appendix 1 for fragmentation using Covaris® M220 Focused-ultrasonicator™. The settings below are specific to Diagenode Bioruptor® Pico, for other models please refer to the manufacturer's protocol.

1. Cool the Bioruptor Pico to 4˚C.
2. Transfer 200 ng of purified DNA to a 0.1 mL Bioruptor tube using up to 50 µl of sample. If less than 50 µl of sample is used, bring the total volume in the Bioruptor tube to 50 µl using **TE Buffer pH 8.0**.
3. Chill DNA in Bioruptor tube for 10 min on ice.
4. Fragment the sample as follows:
   Fragment 4 cycles of 30 sec ON/30 sec OFF
   Remove tube from carousel. Quick spin and pipet up and down 10 times to mix.
   Fragment 4 cycles of 30 sec ON/30 sec OFF
5. Check the size distribution of the fragmented sample on a TapeStation or Bioanalyzer. The fragment size distribution should be centered around 350 bp (see example below).
6. If the average fragment size is larger than desired, re-fragment sample for 3 more cycles of 30 sec ON/30 sec OFF and confirm the size distribution of the re-fragmented sample. Repeat as needed to reach the desired size distribution.



Electropherogram of sufficiently fragmented DNA sample, analyzed with Agilent HS D1000 ScreenTape.

STOP The fragmented DNA can be stored at -20˚C for up to 6 months.

Stage 3. Library Preparation

If you have purchased the add-on modules: Dovetail™ Library Module for Illumina® and Dovetail™ Primer Set for Illumina®, please follow the library preparation protocol below. If you are using one of the following third-party library preparation kits: NEBNext® II DNA Library Prep Kit for Illumina®, Kapa® Hyper Prep Kit, or Swift Accel-NGS® 2S Plus DNA Library Kit, please stop here and follow the instructions of the library preparation kit you purchased.

The Ligation Capture & Amplification Stage (Stage 4) is still required when using a third-party library preparation kit.
Following the library preparation method of your choice, please proceed to
Stage 4. Ligation Capture & Amplification on page 26.

I. End Repair (Box 2 & Dovetail™ Library Module for Illumina®)

NOTE: The End Repair Buffer may have precipitated in storage. Incubate for at least 10 min at 37 ˚C until there is no visible precipitate.

1. Place in a 0.2 mL PCR tube:
   - 48 µl Fragmented Sample
   - 7 µl **End Repair Buffer**
   - 3 µl **End Repair Enzyme Mix**
   - 0.5 µl **250 mM DTT**
2. Pipet up and down 10 times to mix. Quick spin the tube.
3. Incubate for 30 min at 20˚C, followed by 30 min at 65˚C, in a thermal cycler. Hold at 12˚C.

II. Adapter Ligation & USER Digest (Dovetail™ Library Module for Illumina®)

1. Add to the 0.2 mL PCR tube containing 58.5 µl of end-repaired sample:
   - 2.5 µl **Adaptor for Illumina**
   - 1 µl **Ligation Enhancer**
   - 30 µl **Ligation Enzyme Mix**
2. Pipet up and down 10 times to mix. Quick spin the tube.
3. Incubate for 15 min at 20˚C, in a thermal cycler. Hold at 12˚C.
3. Following incubation, add 3 µl of **USER Enzyme Mix** to the PCR tube.
4. Pipet up and down 10 times to mix. Quick spin the tube.
5. Incubate for 15 min at 37˚C, in a thermal cycler. Hold at 12˚C.

III. DNA Purification (Box 1)

1. Vortex **SPRIselect™ Beads** for 30 sec to resuspend.

2. Add 80 µl of the resuspended **SPRIselect™ Beads** to the PCR tube.
3. Vortex to resuspend, quick spin and incubate for 5 min at room temperature off magnet.
4. Quick spin the tube and place on the magnet for 5 min. Remove supernatant.
5. Leave tube on the magnet, and wash beads twice with 100 µl **80% EtOH**. Do not resuspend the beads for these washes.
6. After the second wash, quick spin the tube and place on the magnet for 1 min. Use a pipet with a fine tip to remove the last EtOH traces.
7. Air dry the beads for 5 min on the magnet until no residual EtOH remains. Do not over dry.
8. Off magnet, resuspend beads in 100 µl **TE Buffer pH 8.0**.
9. Vortex briefly, quick spin and incubate for 5 min at room temperature off magnet.
10. Quick spin the tube and place on the magnet for 1 min.
11. **Transfer 95 µl of the SUPERNATANT to a new tube.** Discard Beads.

Purified DNA sample can be stored at -20˚C overnight.

Stage 4. Ligation Capture & Amplification

I. Streptavidin Beads Preparation (Box 1)

NOTE: Does not involve any DNA sample.

1. Vortex **Streptavidin Beads** thoroughly to resuspend. Transfer 25 µl of the resuspended **Streptavidin Beads** to a fresh 1.5 mL tube.
2. Place tube containing the Streptavidin Beads on the magnet for 5 min. Remove supernatant.
3. Remove the tube from the magnetic rack, wash **Streptavidin beads** with 200 µl **TWB (Red Label)**: pipet up and down 10 times to resuspend the beads then place tube on the magnet for 1 min. Remove supernatant.
4. Repeat step 3 for a second wash.
5. After the second wash, resuspend the **Streptavidin Beads** in 100 µl **2X NTB (Yellow Label)**. Pipet up and down 10 times to mix.

II. Ligation Capture (Box 1)

1. Transfer 95 µl of the purified DNA to the 1.5 mL tube containing the **Streptavidin Beads** resuspended in 100 µl of **2X NTB**.
2. Vortex for 10 seconds to thoroughly mix. Quick spin tube.
3. Incubate for 30 min at 25˚C, in an agitating thermal mixer (1250 rpm).

III. Wash Sample on Streptavidin Beads (Box 1)

NOTE: For each of the washes below: remove the tube from the magnetic rack, add the indicated buffer to the beads, pipet up and down 10 times to resuspend the beads then place tube on the magnet for 1 min and remove supernatant (**remove all of the supernatant between each wash; residual supernatant can interfere with the downstream PCR).**

1. Quick spin tube and place on magnet for 1 min. Remove supernatant.
2. Wash beads once with 200 µl **LWB (Green Label)**
3. Wash beads twice with 200 µl **NWB (Blue Label)**
4. Wash beads twice with 200 µl **Wash Buffer.**

IV. Index PCR (Box 2, Dovetail™ Library Module for Illumina® & Dovetail™ Primer Set for Illumina®)

NOTE: Not all PCR enzymes and master mixes are compatible for amplification in the presence of Streptavidin beads. Please use the PCR ready mix supplied in your Dovetail Hi-C Kit (Box 2).

1. After the last wash has been removed, remove the tube from the magnetic rack then add to beads:
   25 µl **HotStart PCR Ready Mix**
   5 µl **Universal PCR Primer**
   5 µl **Index Primer** (unique to each sample, see appendix 2 for list of index primers)
   15 µl **DNase and RNase-free distilled water**
2. Pipet up and down 10 times to resuspend then transfer to a fresh 0.2 mL PCR tube.
3. Quick spin the tube and place it into the thermal cycler. Run the following program:

Temperature	Time	Cycles
98˚C	3 min	---
98˚C	20 sec	11 cycles
65˚C	30 sec	
72˚C	30 sec	
72˚C	1 min	---
12˚C	Hold	

V. Size Selection

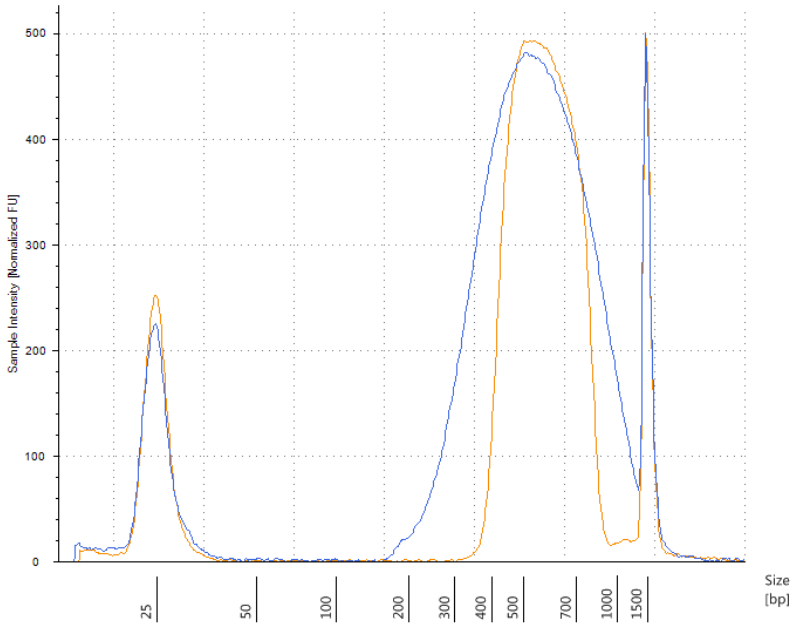
NOTE: We recommend size selecting the Hi-C library, with a size range of 350 to 850 bp.
There are two methods for size selection: one using Sage Science Pippin Prep (recommended method) and one using SPRIselect™ beads.
If you intend to size select the library using Sage Science Pippin Prep, please follow Option A. If you intend to size select the library using SPRIselect™ beads, please follow Option B.

Option A.

NOTE: 1.5% Agarose, Dye-free, Marker K cassette (Sage Science, CDF1510) is recommended.

1. Quick spin the PCR tube and place on the magnet for 1 min.
2. **Transfer 50 µl of the SUPERNATANT to a new 1.5 mL tube.** Discard beads.
3. Vortex **SPRIselect™ Beads** for 30 sec to resuspend.
4. Add 100 µl of resuspended **SPRIselect™ Beads** to the 1.5 mL sample tube.
5. Vortex to resuspend, quick spin and incubate for 5 min at room temperature off magnet.
6. Quick spin the tube and place on the magnet for 5 min. Remove supernatant.
7. Leave tube on the magnet, and wash beads twice with 200 µl **80% EtOH**. Do not resuspend the beads for these washes.
8. After the second wash, quick spin the tube and place on the magnet for 1 min. Use a pipet with a fine tip to remove the last EtOH traces.
9. Air dry beads for 5 min on the magnet until no residual EtOH remains. Do not over dry.
10. Off magnet, resuspend beads in 45 µl **TE Buffer pH 8.0**.
11. Vortex briefly, quick spin and, incubate for 5 min at room temperature off magnet.

12. Quick spin the tube and place on the magnet for 1 min.
13. Transfer 43 µl of the **SUPERNATANT to a new 1.5 mL tube. The tube containing the supernatant is now the pre-selected Hi-C library.**
14. Quantify your pre-selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit. If you recover:
    - Less than 40 ng: discard the library.
    - Between 40 ng and 100 ng: skip the size selection step and check the size distribution of your library on a TapeStation or Bioanalyzer. If there are sharp peaks <200 bp, it can indicate adaptor/primer dimers and can affect sequencing results. In this case, discard the library.
    - > 100 ng, size select the library using Sage Science Pippin Prep according to the manufacturer's instructions. Verify the size distribution of your size-selected library (example below).

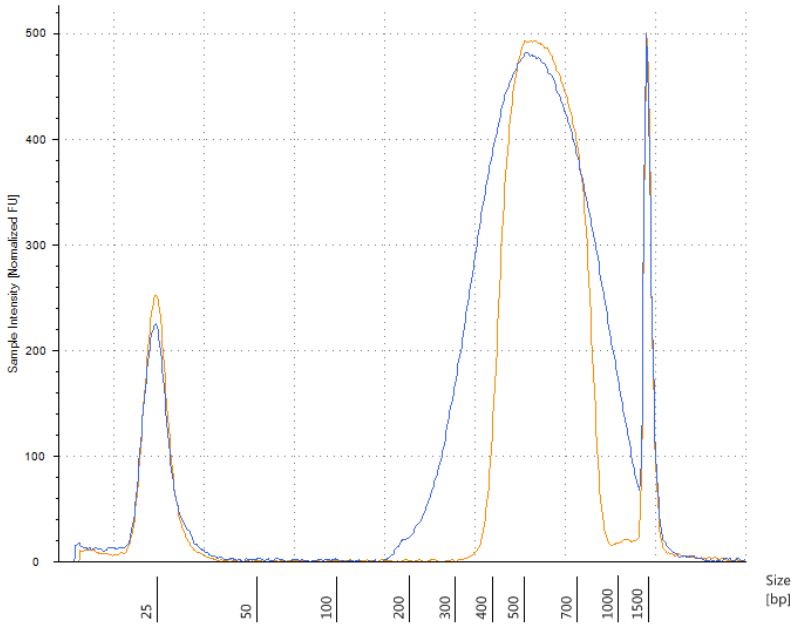


Example of size distribution of a library before (in blue) and after (in orange) size selection, as visualized on a TapeStation D1000.

STOP You can store the library at -20˚C for up to 6 months.

Option B.

1. Quick spin the PCR tube and place on the magnet for 1 min.
2. **Transfer 47 µl of the SUPERNATANT to a new 1.5 mL tube.** Discard beads.
3. Bring the volume of the sample in the 1.5 mL tube to 100 µl using **TE Buffer pH 8.0**.
4. Vortex **SPRIselect™ Beads** for 30 sec to resuspend.
5. Add 55 µl of resuspended **SPRIselect™ Beads** to the 1.5 mL sample tube.
6. Vortex to resuspend, quick spin and incubate for 10 min at room temperature off magnet.
7. Quick spin the tube and place on the magnet for 5 min.
8. **Transfer 155 µl of the SUPERNATANT to a new 1.5 mL tube.** Discard beads.
9. Add 25 µl of resuspended SPRIselect™ Beads to the 1.5 mL tube.
10. Vortex to resuspend, quick spin and incubate for 10 min at room temperature off magnet.
11. Quick spin the tube and place on the magnet for 5 min. Remove supernatant.
12. Leave tube on the magnet, and wash beads twice with 200 µl **80% EtOH**. Do not resuspend the beads for these washes.
13. Quick spin the tube and place on the magnet for 1 min. Use a 10 µl pipet tip to remove traces of EtOH.
14. Air dry beads for 5 min on the magnet until no residual EtOH remains. Do not over dry.
15. Off magnet, resuspend beads in 20 µl **TE Buffer pH 8.0.**
16. Pipet up and down 10 times to resuspend. Quick spin and incubate for 2 min at room temperature off magnet.
17. Quick spin the tube and place on the magnet for 1 min.
18. **Transfer 16 µl of the SUPERNATANT to a new 1.5 mL tube. The tube containing the supernatant is your size selected library.** Discard the beads.
19. Use a TapeStation or Bioanalyzer to verify the size distribution of your size-selected library (example below)



Example of size distribution of a library before (in blue) and after (in orange) size selection, as visualized on a TapeStation D1000.

STOP You can store the library at -20˚C for up to 6 months.

Appendix 1: Fragmentation Using Covaris® M220 Focused-ultrasonicator™

1. Transfer 200 ng of purified DNA to a Covaris® microTUBE AFA Fiber Snap-Cap (part no. 520045) using up to 55 µl of sample. If less than 55 µl of sample is used, bring the total volume to 55 µl using **TE Buffer pH 8.0**.
2. Fragment at the following settings:
    a. Peak Incident Power (W): 50
    b. Duty Factor: 20%
    c. Cycles per Burst: 200
    d. Treatment time (s): 100
    e. Temperature (˚C): 20
    f. Sample volume (µl): 50
3. Check the size distribution of the fragmented sample on a TapeStation or Bioanalyzer. The fragment size distribution should be centered around 350 bp. Re-fragment sample if needed.

The fragmented DNA can be stored at -20˚C for up to 6 months.

Appendix 2: Index Primers

Dovetail™ Primer Set for Illumina® includes the following eight index primers.

Index Primer	Sequence
Index Primer 2	CGATGT
Index Primer 4	TGACCA
Index Primer 5	ACAGTG
Index Primer 6	GCCAAT
Index Primer 7	CAGATC
Index Primer 8	ACTTGA
Index Primer 12	CTTGTA
Index Primer 19	GTGAAA

To choose which index primers to use for multiplexing, please refer to the table below:

Number of Libraries	Index Primer Combination
2	6 and 12
2	5 and 19
3	2, 7 and 19
3	Either of the 2-plex options plus any other Index Primer
4	5, 6, 12 and 19
4	Either of the 3-plex options plus any other Index Primer