Dovetail
GENOMICS

Dovetail™ HiChIP *MNase* Kit

User Manual
V 1.0

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Dovetail™ HiChIP *MNase* Kit Components & Storage

Each kit contains a sufficient supply of materials to perform 8 reactions. The Dovetail™ HiChIP *MNase* Kit comes as two boxes. Store the boxes as listed below immediately upon receipt.

Dovetail™ Chromatin Immunoprecipitation Core

Box 1

(PN DG-CHP-001)

Components	Color	Storage
TE Buffer pH 8.0	None	2°C to 8°C
10X Wash Buffer	White label	
TWB Solution		
2X NTB Solution		
LWB Solution		
NWB Solution		
Protein A/G Beads		
10X Crosslink Reversal Buffer		
Streptavidin Beads		
10X RIPA		
20% SDS		

Dovetail™ Micro-C Module

Box 2

(PN DG-NUC-001)

Components	Color	Storage
MNase Enzyme Mix		-30°C to -10°C
10X Nuclease Digest Buffer		
100 mM $MgCl_2$		
0.5 M EGTA		
End Polishing Enzyme Mix		
End Polishing Buffer		
5X Bridge Ligation Buffer		
Bridge Ligase (T4 DNA Ligase)		
Bridge		
Intra-Aggregate Ligation Enzyme Mix		
Intra-Aggregate Ligation Buffer		
Proteinase K		
250 mM DTT		
HotStart PCR Ready Mix		

Optional Add-on Modules: Components & Storage

Dovetail™ Primer Set For Illumina (PN DG-PRS-001)		
Components	**Color**	**Storage**
Index Primers (x 8, different)		-30°C to -10°C
Universal PCR Primer		

Dovetail™ Library Module For Illumina (PN DG-LIB-001)		
Components	**Color**	**Storage**
End Repair Enzyme Buffer		-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

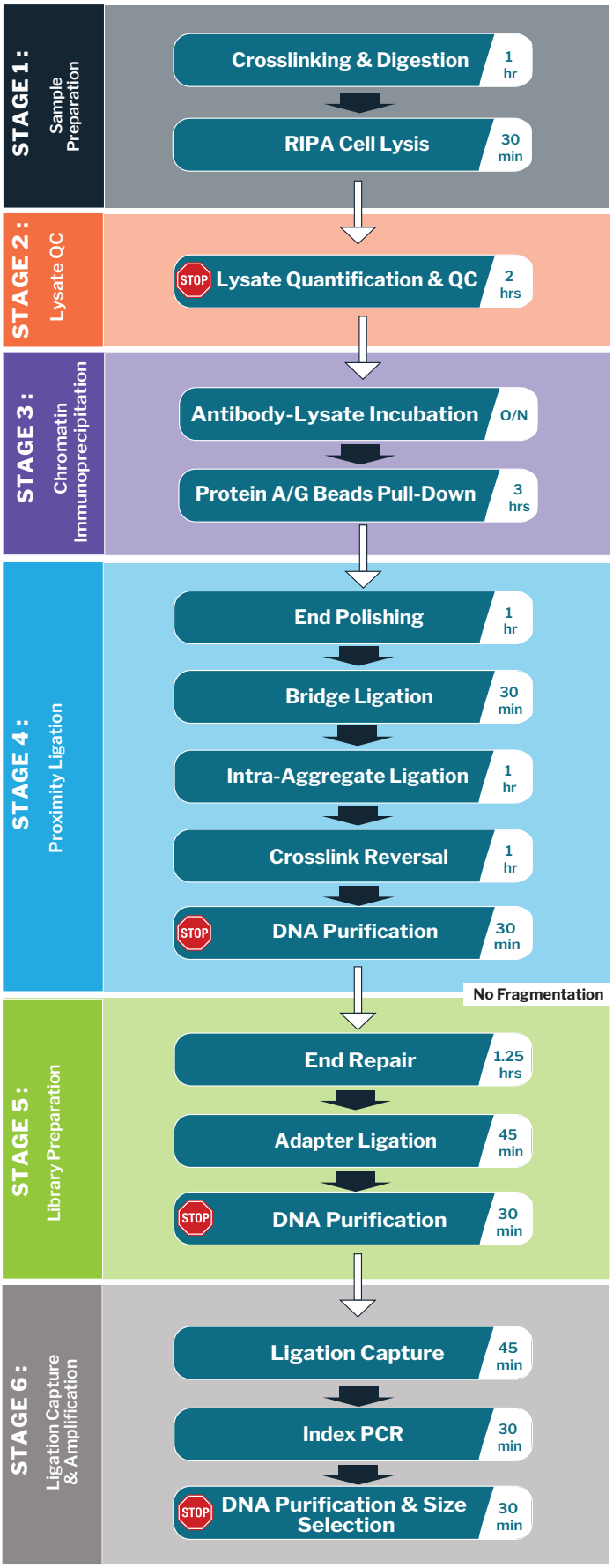
Reagents

Reagents	Supplier	PN
SPRIselect™ Beads, 5 mL	Beckman Coulter	B23317
37% Formaldehyde Solution	Sigma-Aldrich	B23317
1X PBS, pH 7.4, 500 mL	Thermo Fisher Scientific	10010023
100% EtOH	Generic	N/A
UltraPure™ DNase / RNase-Free Distilled Water, 500 mL	Thermo Fisher Scientific	10977015
Zymo DNA Clean & Concentrator-5	Zymo Research	D4013
DSG (Disuccinimidyl Glutarate)	Thermo Fisher Scientific	A35392
DMSO (Dimethyl Sulfoxide, Anhydrous ≥ 99.99%)	Sigma-Aldrich	276855-100ML
cOmplete™ Protease Inhibitor Cocktail	Sigma-Aldrich	11697498001

Consumables and Equiptment

Reagents	Supplier	PN
1.5 mL Low binding microcentrifuge tubes	Generic	
0.2 mL PCR tubes		
5.0 mL centrifuge tubes		
Pipets and pipet tips		
Magnetic separation rack for 0.2 mL and 1.5 mL tubes		
Agitating thermal mixer		
Thermal cycler		
Vortex mixer		
Centrifuge for 0.2 mL, 1.5 mL and 5 mL tubes		
Hemocytometer		
Qubit® Fluorometer	Thermo Fisher Scientific	Q33226
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854
Qubit® Assay Tubes	Thermo Fisher Scientific	Q32856
TapeStation System(Fragment Analyzer or Bioanalyzer)	Agilent	Various

Dovetail™ HiChIP *MNase* Protocol Overview



Stage 1: Sample Preparation (Crosslinking, Digestion & Lysis)

Getting Started

- Sample preparation should take 2 hours.
- Follow this protocol for sample preparation for 1 x 10^6 cells when using a Dovetail™ validated antibody (IgG, CTCF, H3K4ac, H3K4me3, H3K14ac, H3K27ac, H3K27me3, H3K36me3, SMC3). For non-Dovetail™ validated antibodies, follow Appendix 1 for 10 x 10^6 cells.
- The 10X Wash Buffer, 10X Crosslink Reversal Buffer, and 20% SDS might have precipitated in storage. Please incubate the solutions at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.
- Dilute 10X Wash Buffer to 1X with UltraPure™ Water. Store at room temperature. You will need to prepare ~6 mL of 1X Wash Buffer per sample for the entire protocol. 1X Wash Buffer is stable at room temperature for 2 months.
- Prepare 0.3 M DSG in DMSO (anhydrous) by dissolving 1 mg of DSG in 10.22 µL DMSO. DSG is water-insoluble and moisture-sensitive. Prepare **immediately** before use. Do not store DSG in solution.
- Prepare 25X Proteinase Inhibitors by dissolving 1 tablet of cOmplete™ Protease Inhibitor Cocktail in 2 mL of UltraPure™ Water and place on ice. The 25X Proteinase Inhibitors solution is stable at 4°C for 2 weeks.
- Agitating thermal mixer should be set at 1,250 rpm for 1.5 mL tubes.
- Use good laboratory practices including keeping enzymes on ice prior to use, thawing buffers on ice and vortexing prior to use.
- 1X Nuclease Digest Buffer should be prepared fresh and stored at room temperature. 1X Nuclease Digest Buffer is stable for 1 day at room temperature. You need to prepare 50 µL of 1X Nuclease Digest Buffer per sample. To prepare 1X Nuclease Digest Buffer, mix the following components:

10X Nuclease Digest Buffer	5 µL
100 mM $MgCl_2$	5 µL
UltraPure Water	40 µL
Total volume	50 µL

I. Crosslinking & Digestion

Notes

- **1 x 10^6 cells are needed per HiChIP reaction**
- **Pre-freezing the cells is required to get optimal digestion profile.**
- **All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.**

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot 1 x 10^6 cells into a 1.5 mL tube.
3. Spin the 1 x 10^6 cell aliquot at 3,000 x g for 5 minutes. Carefully remove the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.
5. Thaw your cell pellet at room temperature then resuspend the pellet in:
   1 mL 1X PBS
   10 µL 0.3 M DSG
6. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
7. Add 27 µL 37% formaldehyde.
8. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
9. Spin the tube at 3,000 x g for 5 minutes. Carefully remove the supernatant. Use caution, the pellet might be loose.
10. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
11. Spin the tube at 3,000 x g for 5 minutes. Carefully remove the supernatant.
12. Repeat steps 10 and 11 once, for a total of 2 washes.
13. After removing the second wash, resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Getting Started).
14. Add 0.5 µL of MNase Enzyme Mix. Mix by pipetting up and down.
15. Incubate the tube for <u>exactly</u> 15 minutes at 22°C in an agitating thermal mixer set at 1,250 rpm. If you are working with a large number of samples, stagger the start of the digestion for each sample by 20 seconds then stop after corresponding 15 minutes.
16. Stop the reaction by adding 5 µL of 0.5 M EGTA. Mix by pipetting up and down.

II. RIPA Cell lysis

1. Add to the sample tube:
   20 µL 10X RIPA
   8 µL 25X Protease Inhibitors
   1 µL 20% SDS
   115.5 µL UltraPure water
2. Invert the tube to mix then rotate at room temperature for 30 minutes.
3. Spin the tube at 16,000 x g for 2 minutes.
4. Transfer the **SUPERNATANT (lysate)** to a new 1.5 mL tube. This supernatant contains the digested chromatin.
5. Continue to Stage 2: Lysate QC.

Stage 2: Lysate QC

Getting Started

- The Lysate QC stage takes 2 hours.
- The protocol below is written for the TapeStation; however, it is also compatible with the Bioanalyzer System and Fragment Analyzer. Please refer to the table below for our recommended kits for each system.

TapeStation	D5000 HS
Bioanalyzer System	HS DNA
Fragment Analyzer	DNF-488 HS Genomic DNA

- Verify before use that the Zymo™ DNA Wash Buffer contains the appropriate volume of 100% Ethanol, as specified by the manufacturer.

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1. Transfer 10 µL of the lysate to a new 1.5 mL tube labeled QC. Store the remainder of the lysate on ice. This is the lysate you will be using in Stage 3.
2. Add to the QC tube:
   45 µL UltraPure Water
   5 µL 10X Crosslink Reversal Buffer
   1.5 µL Proteinase K
3. Pipet up and down to mix. Incubate the QC tube in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
55°C	15 min
68°C	45 min
25°C	Hold

4. Quick spin your QC tube. Clean up your QC sample using Zymo DNA Clean & Concentrator™-5 kit: start by adding 200 µL of DNA Binding Buffer to your QC tube. Mix thoroughly.
5. Transfer the mixture to the Zymo-Spin™ Column placed in a collection tube.
6. Centrifuge for 30 seconds at 13,000 x g. Discard the flow-through.
7. Add to the column 200 µL Zymo DNA Wash Buffer (see Getting Started).
8. Centrifuge for 1 minute at 13,000 x g. Discard the flow-through.
9. Repeat steps 7 and 8 once, for a total of 2 washes.
10. Transfer the column to a new 1.5 mL tube.
11. Add 10 µL Zymo™ DNA Elution Buffer directly to the column and incubate for 1 minute at room temperature.

12. Centrifuge for 1 minute at 13,000 x g. Discard the column. Your 1.5 mL tube now contains your purified QC DNA.
13. Quantify 5 µL of your purified QC DNA with a Qubit® Fluorometer and Qubit® dsDNA HS Kit. You should recover a minimum of 100 ng.

    Based on your Qubit concentration, your total lysate amount (ng) can be calculated as follows.

    Total Lysate (ng) = Qubit (ng/ µL) x 10 µL (elution volume) x 20 (dilution factor)

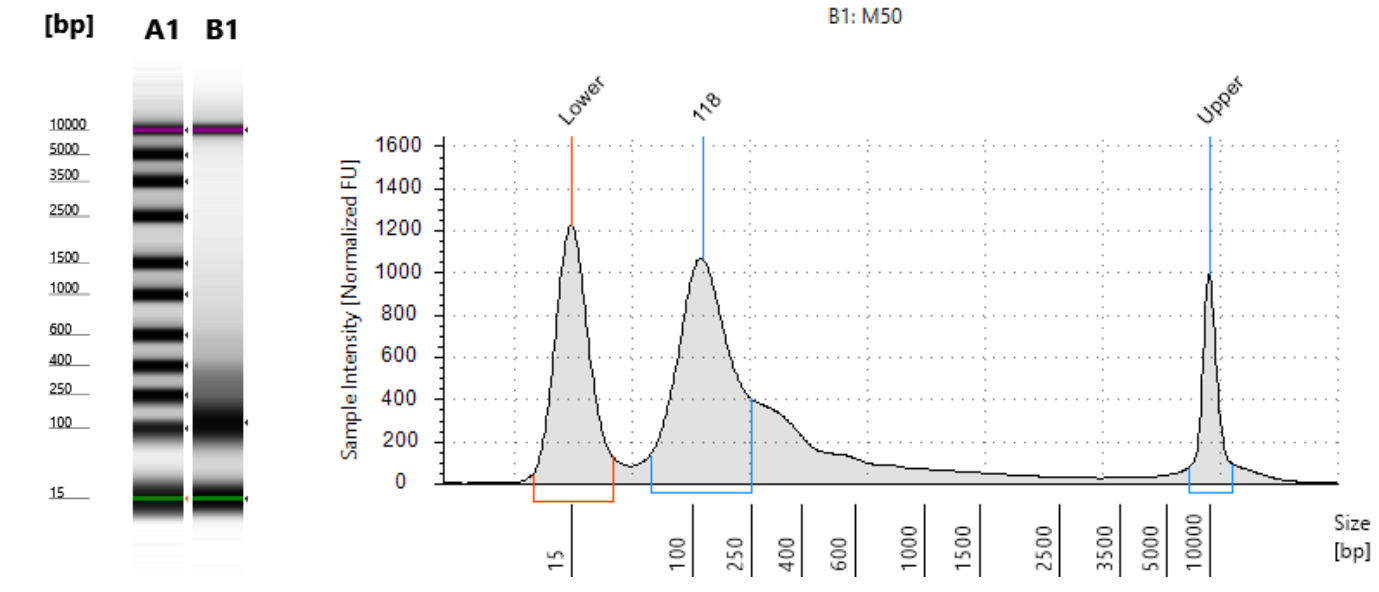
14. Check the fragment size distribution of your purified QC sample on a TapeStation D5000 HS ScreenTape. **Make sure your sample is diluted to 1 ng/µL.**

    - The digestion profile should include at least 30% mononucleosomes: first DNA peak, typically in the size range of 50 - 250 bp for TapeStation, should account for at least 30% of total DNA (Figure 1). The size range of the peak may vary for other analytical instruments such as Bioanalyzer and Fragment Analyzer.

    - If the digestion profile contains < 30% mononucleosomes, do not proceed with the rest of the protocol. In this case, please re-start the protocol and extend the MNase digestion time (Stage 1, Section I, Step 15) by 45 minutes for a total of 1 hour digest time.

Figure 1. Expected (QC Pass) digestion profile, as analyzed on HS D5000 ScreenTape.

QC PASS



Stage 3: Chromatin Immunoprecipitation

I. Antibody-Lysate Incubation

Notes

The volume of antibody to add to the lysate is dependent on the antibody of interest.

Dovetail™ Validated Antibody	Supplier	PN	Volume to add per 100 ng of lysate
IgG	Cell Signaling	2729	2 µL
CTCF	Cell Signaling	3418	2.5 µL
H3K4ac	Active Motif	39381	2.5 µL
H3K4me3	Cell Signaling	9751	2.5 µL
H3K14ac	Cell Signaling	7627	2.5 µL
H3K27ac	Cell Signaling	8173	2.5 µL
H3K27me3	Cell Signaling	9733	2.5 µL
H3K36me3	Cell Signaling	4909	2.5 µL
SMC3	Abcam	Ab9263	2 µL

1. Add the appropriate amount of antibody to the remaining lysate from Stage 2, Step 1.
2. Invert to mix then rotate at 4°C overnight (at least 12 hours).

II. Protein A/G beads Pull-down

Notes

Dilute 10X RIPA to 1X with UltraPure Water. Store at room temperature. You need to prepare ~4 mL of 1X RIPA per sample. 1X RIPA is stable at room temperature for 2 months.

1. Vortex the Protein A/G beads for > 30 seconds to fully resuspend. Quick spin the tube.
2. Place 25 µL of Protein A/G beads in a new 1.5 mL tube.
3. Place the tube in the magnetic rack for 5 minutes or until the solution looks clear and the beads have fully separated. Remove the supernatant.
4. Remove the tube from the magnetic rack and wash the beads with 200 µL 1X RIPA. Pipet up and down to resuspend the beads and place the tube in the magnetic rack for 2 minutes. Remove the supernatant.

5. Repeat step 4 once, for a total of 2 washes.
6. After the last wash has been aspirated, remove the tube from the magnetic rack and resuspend the beads in 50 µL 1X RIPA.
7. Quick spin your antibody-lysate complex that was incubated overnight (Stage 3, Section I, Step 2) and add it to the resuspended beads. Pipet up and down to mix.
8. Rotate at room temperature for 2 hours.
9. Quick spin the tube and place it in the magnetic rack for 2 minutes. Remove the supernatant.
10. Wash the beads with 1 mL 1X RIPA: pipet up and down to resuspend the beads, rotate the tube for 5 minutes, quick spin and place the tube in the magnetic rack for 2 minutes. Remove the supernatant.
11. Repeat step 10 twice, for a total of 3 washes.
12. Wash the beads with 1 mL 1X Wash Buffer: pipet up and down to resuspend the beads, rotate the tube for 5 minutes, quick spin and place the tube in the magnetic rack for 2 minutes. Remove the supernatant.
13. Repeat step 12 twice, for a total of 3 washes.

Stage 4: Proximity Ligation

Getting Started

- Proximity ligation takes 5 hours.
- Agitating thermal mixer should be set at 1,250 rpm for 1.5 mL tubes.
- When placing the sample in the magnetic rack, always wait until the solution is clear to allow the beads to fully separate before removing the supernatant.
- 80% ethanol should be freshly prepared for DNA purification with SPRIselect™ Beads.

I. End Polishing

1. After the last wash has been aspirated, remove the tube from the magnetic rack and add to the beads 53.5 µL of a master mix containing the following reagents:

   50 µL End Polishing Buffer
   3.5 µL End Polishing Enzyme Mix

2. Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
22°C	30 min
65°C	30 min

3. Allow the tube to reach room temperature then place it in the magnetic rack for 1 minute (or until the solution looks clear). Remove the supernatant.
4. Remove the tube from the magnetic rack and wash the beads once with 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads, place the tube in the magnetic rack for 1 minute and remove the supernatant. Remove the tube from the magnetic rack.

II. Bridge Ligation

1. Prepare fresh 50 µL Bridge Ligation Mix by mixing the following components:

   10 µL 5X Bridge Ligation Buffer
   5 µL Bridge
   35 µL UltraPure Water

2. Add to the beads 51 µL of a master mix containing the following reagents:
   50 µL Bridge Ligation Mix
   1 µL Bridge Ligase
3. Pipet up and down to fully mix. Incubate for 30 minutes at 22°C in an agitating thermal mixer set at 1,250 rpm.
4. Place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Remove the supernatant.
5. Remove the tube from the magnetic rack and wash the beads once with 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads, place the tube in the magnetic rack for 1 minute and remove the supernatant.

III. Intra-Aggregate Ligation

1. Remove the tube from the magnetic rack and add to the beads 52 µL of a master mix containing the following reagents:
   50 µL Intra-Aggregate Ligation Buffer
   2 µL Intra-Aggregate Ligation Enzyme Mix
2. Pipet up and down to fully mix. Incubate for 1 hour at 22°C in an agitating thermal mixer set at 1,250 rpm.
   For convenience, this ligation reaction can proceed overnight at 22°C in an agitating thermal mixer set at 1,250 rpm.
3. Place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Remove the supernatant.

IV. Crosslink Reversal

1. Remove the tube from the magnetic rack and add to the beads 51.5 µL of a master mix containing the following reagents:
   45 µL Ultra Pure Water
   5 µL 10X Crosslink Reversal Buffer
   1.5 µL Proteinase K
2. Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
55°C	15 min
68°C	45 min
25°C	Hold

For convenience, you can hold at 25°C overnight in an agitating thermal mixer set at 1,250 rpm.

3. Place the tube in the magnetic rack for 1 minute. Transfer 50 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.

V. DNA Purification on SPRIselect Beads

1. Vortex SPRIselect™ Beads for > 30 seconds to resuspend.
2. Add 90 µL of resuspended SPRIselect™ Beads to the 1.5 mL tube containing your sample.
3. Vortex to resuspend, quick spin and incubate for 5 minutes at room temperature off the magnetic rack.
4. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Remove the supernatant.
5. Leave the tube in the magnetic rack and wash the beads twice with 150 µL 80% EtOH. Do not resuspend the beads for these washes. Simply add the EtOH, wait for 1 minute then remove the EtOH wash.
6. After the second wash, quick spin the tube and place in the magnetic rack for 1 minute. Use a pipet with a fine tip to remove the last EtOH traces.
7. Air dry the beads for 5 minutes in the magnetic rack until no residual EtOH remains. Do not over dry.
8. Off the magnetic rack, resuspend the beads in 57 µL TE Buffer pH 8.0.
9. Vortex briefly, quick spin and incubate for 5 minutes at room temperature off the magnetic rack.
10. Quick spin the tube and place in the magnetic rack for 1 minute. Transfer 55 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.
11. Quantify 5 µL of your sample (purified DNA) using a Qubit Fluorometer and Qubit dsDNA HS Kit. The amount of DNA recovered is dependent on the antibody used, it can be below detection limit.
    The amout of DNA recovered after purification determines the adaptor dilution during library preparation (Stage 5) and number of PCR cycles (Stage 6). This purified DNA will go into library preparation and will be referred to as DNA input.

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

Stage 5: Library Preparation

Getting Started

- The library preparation protocol does not require fragmentation.
- The library preparation protocol takes 2 hours.

I. End Repair

Notes

- **The End Repair Buffer may have precipitated in storage. Incubate for at least 10 minutes at 37˚C until there is no visible precipitate.**
- **Pipet up and down to fully mix 250 mM DTT prior to use.**

1. Place the 50 µL of purified DNA input in a 0.2 mL PCR tube.
2. Add to the PCR tube 10.5 µL of a master mix containing the following reagents:

   7 µL End Repair Buffer
   3 µL End Repair Enzyme Mix
   0.5 µL 250 mM DTT
3. Pipet up and down to mix. Quick spin the tube.
4. Incubate in a thermal cycler as follows:

Temperature	Time
20˚C	30 min
65˚C	30 min
12˚C	Hold

II. Adapter Ligation & USER Digest

1. Determine whether adaptor dilution is necessary. If DNA input is ≤ 5 ng, dilute the Adaptor for Illumina in 10 mM Tris-HCl, pH 7.5 containing 10 mM NaCl, as indicated below.

Input	Adaptor Dilution
500 ng – 5 ng	No Dilution
< 5 ng	2.5-fold (1:2.5)

2. Add to the 0.2 mL PCR tube containing 60.5 µL of end-repaired sample:
   - 2.5 µL Adaptor for Illumina
   - 1 µL Ligation Enhancer
   - 30 µL Ligation Enzyme Mix
3. Pipet up and down to mix. Quick spin the tube.
4. Incubate for 15 minutes at 20˚C in a thermal cycler. Hold at 12˚C.
5. Following incubation, add 3 µL of USER Enzyme Mix to the PCR tube.
6. Pipet up and down to mix. Quick spin the tube.
7. Incubate for 15 minutes at 37˚C in a thermal cycler. Hold at 12˚C.

III. DNA Purification

1. Vortex SPRIselect Beads for 30 seconds 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 minutes at room temperature off the magnetic rack.
4. Quick spin the tube and place in the magnetic rack for 5 minutes. Remove the supernatant.
5. Leave the tube in the magnetic rack and wash the beads twice with 150 µL 80% EtOH. Do not resuspend the beads for these washes.
6. After the second wash, quick spin the tube and place in the magnetic rack for 1 minute. Use a pipet with a fine tip to remove the last EtOH traces.
7. Air dry the beads for 5 minutes in the magnetic rack until no residual EtOH remains. Do not over dry.
8. Off the magnetic rack, resuspend the beads in 100 µL TE Buffer pH 8.0.
9. Vortex briefly, quick spin and incubate for 5 minutes at room temperature off the magnetic rack.
10. Quick spin the tube and place in the magnetic rack for 1 minute.
11. Transfer 95 µL of the **SUPERNATANT** to a new tube. Discard the beads.

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

Stage 6: Ligation Capture & Amplification

Getting Started

The Ligation Capture & Amplification protocol should take 2 hours.

I. Streptavidin Beads Preparation

Notes

This step does not involve any DNA sample.

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

II. Ligation Capture

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 the tube.
3. Incubate for 30 minutes at 25˚C in an agitating thermal mixer set at 1,250 rpm.

III. Wash Sample on Streptavidin Beads

Notes

For each of the washes below, remove the tube from the magnetic rack, add the indicated buffer to the beads, pipet up and down to resuspend the beads, place the tube in the magnetic rack for 1 minute, and remove the supernatant.
Remove all of the supernatant between each wash; residual supernatant can interfere with the downstream PCR.

1. Quick spin the tube and place in the magnetic rack for 1 minute. Remove the supernatant.
2. Wash the beads once with 200 µL LWB (Green Label).
3. Wash the beads twice with 200 µL NWB (Blue Label).
4. Wash the beads twice with 200 µL 1X Wash Buffer.

IV. Index PCR

Notes

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 Kit (Box 2).

1. After the last wash has been aspirated, remove the tube from the magnetic rack and add to the 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 UltraPure DNase and RNase-Free Distilled Water
2. Pipet up and down to resuspend then transfer to a new 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	12 cycles if DNA input (Stage 4, Section 5, Step 11) ≥ 100 ng 14 cycles if DNA input (Stage 4, Section 5, Step 11) < 100 ng - 5 ng 16 cycles if DNA input (Stage 4, Section 5, Step 11) < 5 ng
65°C	20 sec	
72°C	30 sec	
72°C	1 min	
12°C	Hold	

V. Size Selection

1. Quick spin the PCR tube and place it in the magnetic rack for 1 minute.
2. Transfer 47 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the 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 seconds to resuspend.
5. Add 50 µL of resuspended SPRIselect Beads to the 1.5 mL tube containing your sample.
6. Vortex to resuspend, quick spin and incubate for 10 minutes at room temperature off the magnetic rack.

7. Quick spin the tube and place in the magnetic rack for 5 minutes.
8. Transfer 145 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.
9. Add 30 µL of resuspended SPRIselect Beads to the 1.5 mL tube.
10. Vortex to resuspend, quick spin and incubate for 10 minutes at room temperature off the magnetic rack.
11. Quick spin the tube and place in the magnetic rack for 5 minutes. Remove the supernatant.
12. Leave the tube in the magnetic rack and wash the beads twice with 200 µL 80% EtOH. Do not resuspend the beads for these washes.
13. Quick spin the tube and place in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of EtOH.
14. Air dry the beads for 5 minutes in the magnetic rack until no residual EtOH remains. Do not over dry.
15. Off the magnetic rack, resuspend the beads in 30 µL TE Buffer pH 8.0.
16. Pipet up and down to resuspend. Quick spin and incubate for 2 minutes at room temperature off the magnetic rack.
17. Quick spin the tube and place in the magnetic rack for 1 minute.
18. Transfer 28 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. Quantify your size selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit.
20. Use a TapeStation or Bioanalyzer to verify the size distribution of your size-selected library. The size range is expected to be between 350 bp and 1,000 bp.

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

Appendix 1: Sample Preparation (Stage 1) for 10 x 10^6 cell input

Getting Started

- You are following this appendix for non-Dovetail™ validated antibodies that require starting with 10 x 10^6 cell input.
- Sample preparation should take 2 hours.
- The 10X Wash Buffer, 10X Crosslink Reversal Buffer, and 20% SDS might have precipitated in storage. Please incubate the solutions at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.
- Dilute 10X Wash Buffer to 1X with UltraPure Water. Store at room temperature. You will need to prepare ~6 mL of 1X Wash Buffer per sample for the entire protocol. 1X Wash Buffer is stable at room temperature for 2 months.
- Prepare 0.3 M DSG in DMSO (anhydrous) by dissolving 1 mg of DSG in 10.22 µL DMSO. DSG is water-insoluble and moisture-sensitive. Prepare **immediately** before use. Do not store DSG in solution.
- Prepare 25X Proteinase Inhibitors by dissolving 1 tablet of cOmplete™ Protease Inhibitor Cocktail in 2 mL of UltraPure Water and place on ice. The 25X Proteinase Inhibitors solution is stable at 4°C for 2 weeks.
- Agitating thermal mixer should be set at 1,250 rpm for 1.5 mL tubes.
- Use good laboratory practices including keeping enzymes on ice prior to use, thawing buffers on ice and vortexing prior to use.
- 1X Nuclease Digest Buffer should be prepared fresh and stored at room temperature. 1X Nuclease Digest Buffer is stable for 1 day at room temperature. You need to prepare 100 µL of 1X Nuclease Digest Buffer per sample. To prepare 1X Nuclease Digest Buffer, mix the following components:

10X Nuclease Digest Buffer	10 µL
100 mM $MgCl_2$	10 µL
UltraPure Water	80 µL
Total volume	100 µL

I. Crosslinking & Digestion

Notes

- **10 x 10^6 cells are needed per HiChIP reaction**
- **Pre-freezing the cells is required to get optimal digestion profile.**
- **All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.**

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot 10 x 10^6 cells into a 1.5 mL tube.

3. Spin the 10 x 10^6 cell aliquot at 3,000 x g for 5 minutes. Carefully remove the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.
5. Thaw your cell pellet at room temperature then resuspend the pellet in:
   1 mL 1X PBS
   10 µL 0.3 M DSG
6. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
7. Add 27 µL 37% formaldehyde.
8. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
9. Spin the tube at 3,000 x g for 5 minutes. Carefully remove the supernatant. Use caution, the pellet might be loose.
10. Wash the pellet with a total of 1 mL 1X Wash buffer: first add 200 µL of wash buffer and pipet to break up clumps, then add the remaining 800 µL. Pipet up and down to fully resuspend the pellet.
11. Spin the tube at 3,000 x g for 5 minutes. Carefully remove the supernatant.
12. Repeat steps 10 and 11 once, for a total of 2 washes.
13. After removing the second wash, resuspend the cell pellet in 100 µL 1X Nuclease Digest Buffer (freshly prepared, see Getting Started).
14. Add 1 µL of MNase Enzyme Mix. Mix by pipetting up and down.
15. Incubate the tube for <u>exactly</u> 15 minutes at 22°C in an agitating thermal mixer set at 1,250 rpm. If you are working with a large number of samples, stagger the start of the digestion for each sample by 20 seconds then stop after corresponding 15 minutes.
16. Stop the reaction by adding 10 µL of 0.5 M EGTA. Mix by pipetting up and down.

II. RIPA Cell lysis

1. Add to the sample tube:
   20 µL 10X RIPA
   8 µL 25X Protease Inhibitors
   1 µL 20% SDS
   70 µL UltraPure water
2. Invert the tube to mix then rotate at room temperature for 30 minutes.
3. Spin the tube at 16,000 x g for 2 minutes.
4. Transfer the **SUPERNATANT** (lysate) to a new 1.5 mL tube. This supernatant contains the digested chromatin.
5. Continue to Stage 2: QC Lysate.

Appendix 2: Index Primers

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 or 5 and 19
3	2, 7 and 19 or either of the 2-plex options plus any other Index Primer
4	5, 6, 12 and 19 or either of the 3-plex options plus any other Index Primer