Cantata Bio

Dovetail® HiChIP *MNase* Kit

Mammalian Cells

USER GUIDE

VERSION 1.3

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Dovetail® HiChIP *MNase* Kit Components and Storage

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

Table 1. Dovetail® Chromatin Immunoprecipitation Core Box 1 (PN DG-CHIP-001)

Components	Size	Cap Color	Label Color	Storage
TE Buffer pH 8.0	30 mL Bottle			2°C to 8°C
10X Wash Buffer	60 mL Bottle			
TWB Solution	30 mL Bottle			
2X NTB Solution	30 mL Bottle			
LWB Solution	30 mL Bottle			
NWB Solution	30 mL Bottle			
Protein A/G Beads	0.5 mL Tube			
10X Crosslink Reversal Buffer	2 mL Tube			
Streptavidin Beads	0.5 mL Tube			
10X RIPA	15 mL Tube			
20% SDS	0.5 mL Tube			

Table 2. Dovetail® Micro-C Module Box 2 (PN DG-NUC-001)

Components	Tube/Bottle Size	Cap Color	Label Color	Storage
MNase Enzyme Mix	0.5 mL Tube			-30°C to -10°C
10X Nuclease Digest Buffer	2 mL Tube			
100 mM $MgCl_2$	0.5 mL Tube			
0.5 M EGTA	0.5 mL Tube			
End Polishing Enzyme Mix	0.5 mL Tube			
End Polishing Buffer	2 mL Tube			
5X Bridge Ligation Buffer	0.5 mL Tube			
Bridge Ligase	0.5 mL Tube			
Bridge	0.5 mL Tube			
Intra-Aggregate Ligation Enzyme Mix	0.5 mL Tube			
Intra-Aggregate Ligation Buffer	2 mL Tube			
Proteinase K	0.5 mL Tube			
250 mM DTT	0.5 mL Tube			
HotStart PCR Ready Mix	0.5 mL Tube			

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

Optional Add-on Modules: Components and Storage

Dovetail® Dual Index Primer Set #1 For Illumina

Table 3. Dovetail® Dual Index Primer Set #1 For Illumina® (PN DG-PRS-002)

Components	Size	Cap Color	Label Color	Storage
Unique Dual Index (UDI) Primer Pairs (x 8, different)	0.5 mL Tube			-30°C to -10°C

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

Dovetail® Library Module For Illumina

Table 4. Dovetail® Library Module For Illumina (PN DG-LIB-001)

Components	Size	Cap Color	Label Color	Storage
End Repair Enzyme Buffer	0.5 mL Tube			-30°C to -10°C
End Repair Enzyme Mix	0.5 mL Tube			
Ligation Enhancer	0.5 mL Tube			
Ligation Enzyme Mix	0.5 mL Tube			
Adaptor for Illumina	0.5 mL Tube			
USER Enzyme Mix	0.5 mL Tube			

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

User Supplied Reagents, Consumables and Equipment

Reagents

Table 5. Reagents

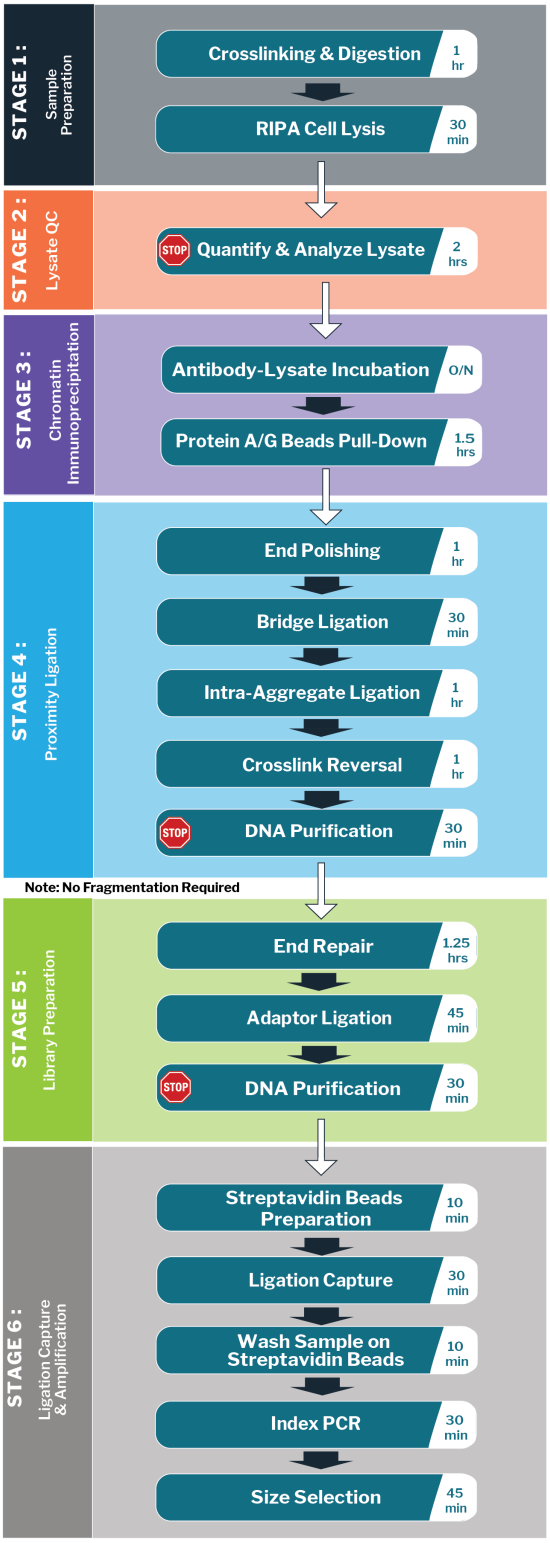
Reagents	Supplier	Part Number
SPRIselect® Beads, 5 mL	Beckman Coulter	B23317
37% Formaldehyde Solution	Sigma-Aldrich	F8775
1X PBS, pH 7.4, 500 mL	Thermo Fisher Scientific	10010023
100% EtOH	Generic	N/A
UltraPure™ DNase / RNase-Free Distilled Water, 500 mL	Thermo Fisher Scientific	10977015
DNA Clean & 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 Equipment

Table 6. Consumables and Equipment

Consumables/Equipment	Supplier	Part Number
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



Good Practices

1. Read the entire guide before use, including Before You Begin and the Notes.
2. The cell input amount will influence the efficiency of the digestion reaction. To ensure an accurate cell count, use best practices such as low-speed spins (< 500 x g) using a swinging bucket rotor when harvesting the cells, and counting prior to freezing.
3. To ensure efficient crosslinking, a new or recently opened solution of formaldehyde should be used. Formaldehyde solution containing white precipitates should not be used.
4. Keep all enzymes and master mixes on ice during setup and use. Promptly move reagents back to the indicated storage.
5. Fully thaw buffers, place on ice and thoroughly mix before use.
6. When preparing master mixes, scale the volume of each reagent as appropriate, using 10% excess volume to compensate for pipetting loss.
7. Always add the reagents to the master mix in the specified order as listed throughout the protocol.
8. When working with beads, such as Protein A/G beads, SPRIselect and Streptavidin beads, you should:
    a. Equilibrate the beads to room temperature before use.
    b. Thoroughly vortex the beads immediately before use and ensure they are a homogenous slurry before use.
    c. When placing the tube in the magnetic rack, always wait until the solution looks clear to allow the beads to fully separate before removing the supernatant carefully and slowly. This helps minimize bead/sample loss throughout the protocol.
    d. Do not let the beads dry out during washing steps. Keep the beads in buffer until ready to resuspend them for the next step.
    e. After washing the SPRIselect beads with 80% ethanol during DNA purification, do not let the beads over-dry before proceeding with elution. Over-drying the beads may result in lower recovery of DNA.

Things to Consider Before You Start

How many cells should you start the assay with?

The amount of cells to use for the HiChIP *MNase* assay can vary significantly depending on the cell type, the cell culture, and the abundance of the protein of interest. Please refer to the table below for the recommended starting cell number for your antibody of interest.

Antibody	Supplier	Part Number	Recommended amount (ng or µL) of antibody	Recommended amount (ng) of chromatin (clarified lysate) input	Corresponding starting cell number
IgG	Cell Signaling	2729	Equivalent amount as the antibody of interest	Equivalent amount as the antibody of interest	Equivalent number as the antibody of interest
CTCF	Cell Signaling	3418	500 ng	1,500	5×10^6
H3K4ac	Active Motif	39381	7.5 µL	1,000	5×10^6
H3K4me3	Cell Signaling	9751	1,250 ng	500	5×10^6
H3K14ac	Cell Signaling	7627	4,000 ng	1,000	5×10^6
H3K27ac	Cell Signaling	8173	300 ng	1,000	5×10^6
H3K27me3	Cell Signaling	9733	1,250 ng	1,000	5×10^6
H3K36me3	Cell Signaling	4909	1,250 ng	1,000	5×10^6
PolII	Abcam	ab26721	20,000 ng	1,500	5×10^6
Klf4	Abcam	ab106629	20,000 ng	10,000	10×10^6
Nanog	Abcam	ab21624	5,000 ng	10,000	10×10^6
Oct4	Cell Signaling	2890	25 µL	10,000	10×10^6
Sox2	Cell Signaling	23064	500 ng	10,000	10×10^6
Non-Dovetail Validated Antibody	N/A	N/A	N/A	N/A	10×10^6

NOTES

- The first time you run the assay with a given cell type, we recommend to process a single cell aliquot through Stage 1 and Stage 2 to ensure the MNase digestion conditions are optimal for your cell type. Once the digestion conditions are optimized for your cell type, they are reproducible so you can set up multiple preparations in parallel.
- If 5 x 10^6 cells are not available, we recommend moving forward with the maximum number of cells obtainable and processing those in 1 x 10^6 cell aliquots according to the 1 x 10^6 cell sample preparation protocol. If you have fewer than 1 x 10^6 cells, there is a high risk of not having sufficient yield to convert into a library or your library will have a high duplication rate and poor ChIP enrichment.

Why is it important to achieve an optimal digestion?

Dovetail HiChIP *MNase* assay does not require sonication prior to library preparation. The fragmentation is solely based on enzymatic digestion with MNase. Achieving an optimal digestion profile which contains 40 – 70% mononucleosomes results in a high complexity library with enriched long-range interactions. Under-digested (< 40% mononucleosomes) or over-digested (> 70% mononucleosomes) chromatin results in lower lysate yield and low complexity library (i.e. high duplication rate). When the chromatin is under-digested, fewer chromatin fragments are captured and proximity ligated. This significantly reduces the long-range interactions captured in the library. When the chromatin is over-digested, the di- and tri-nucleosomes are lost and the long-range interactions are therefore minimized.

How much MNase enzyme should you use to achieve an optimal digestion?

The amount of MNase Enzyme Mix to use depends on the cell type and number.The appropriate MNase amount for your sample input and type may need to be optimized. The amounts provided in the User Guide are a general starting point. If the digestion profile is not optimal, consult Appendix 2: Troubleshooting Guide, page 40 for how to optimize the digestion.

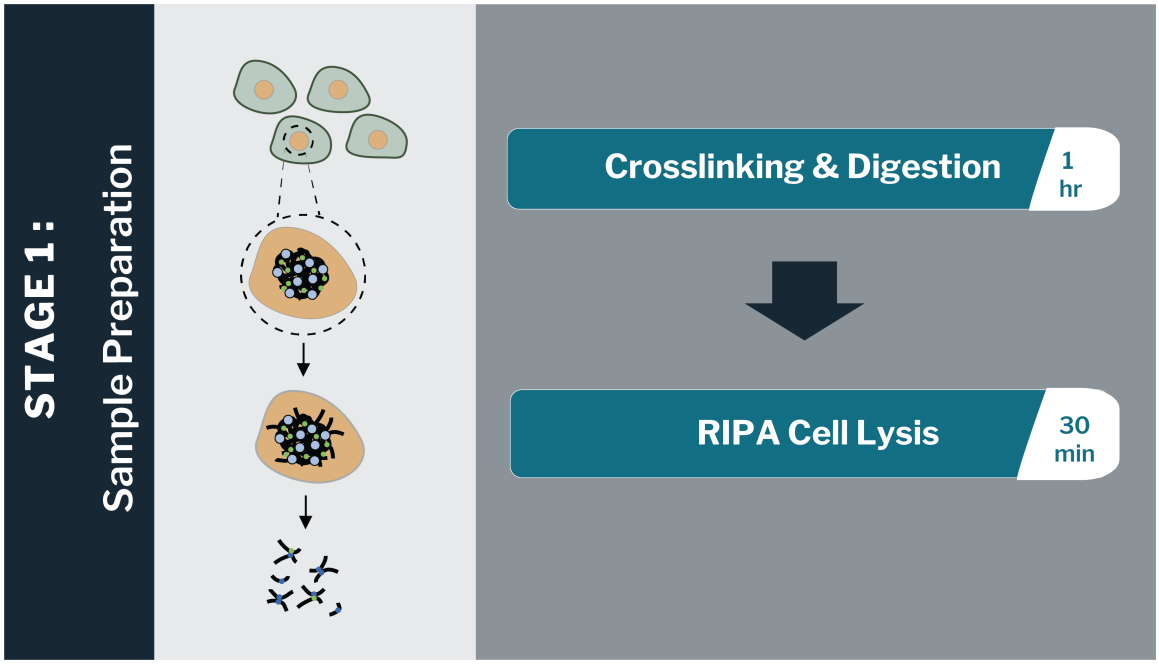
Input	Enzyme Amount
1 x 10^6 cells	0.5 µL of 1:2 diluted MNase Enzyme Mix
5 x 10^6 cells	0.5 µL of undiluted MNase Enzyme Mix
10 x 10^6 cells	1 µL of undiluted MNase Enzyme Mix

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

As you prepare for Stage 1, keep the following in mind:

- Sample preparation takes ~ 1.5 hours.
- Follow this sample preparation protocol if you are starting the assay with 5 x 10^6 cells (Option A) or 10 x 10^6 cells (Option B). If you are starting with 1 x 10^6 cells, follow Appendix 1: Sample Preparation for 1 x 10^6 Cell Input, page 37.

Figure 1. Stage 1: Sample Preparation



Before You Begin

- The 10X Wash Buffer and ● 20% SDS might have precipitated in storage. Incubate these 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. 1X Wash Buffer is stable at room temperature for 2 months. You need ~6 mL of 1X Wash Buffer per sample for the entire protocol.
- 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.

- Prepare fresh 1X Nuclease Digest Buffer and store at room temperature. 1X Nuclease Digest Buffer is stable for 1 day at room temperature. You need 100 µL of 1X Nuclease Digest Buffer per sample. To prepare 1X Nuclease Digest Buffer (100 µL), mix the following components:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	80 µL	88 µL	x	8	=	704 µL
● 10X Nuclease Digest Buffer	10 µL	11 µL	x	8	=	88 µL
● 100 mM $MgCl_2$	10 µL	11 µL	x	8	=	88 µL
Total	**100 µL**					

- Set the thermal mixer at 22°C, shaking at 1,250 rpm.
- Thaw ● 0.5 M EGTA at room temperature. Vortex to mix prior to use.

Option A: If starting the assay with 5 x 10^6 cells

1.1 Crosslinking and Digestion

NOTES

- 5 x 10^6 cells are needed per HiChIP reaction.
- Pre-freezing the cells is required to get an optimal digestion profile.
- The cell pellet should be frozen at -80°C for at least 30 minutes (step 4). You can keep the cells frozen at -80°C for longer as long as they are not subjected to freeze-thaw.
- All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.

Follow the steps below for Crosslinking and Digestion:

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot 5 x 10^6 cells into a 1.5 mL tube.
3. Spin the 5 x 10^6 cell aliquot at 1,000 x g for 5 minutes. Carefully remove and discard the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.
5. Thaw your cell pellet **quickly** 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 of 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 and discard 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 1X Wash Buffer using a 200 µL pipette 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 and discard 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 **Before You Begin, page 12**).
14. Add 0.5 µL of ● MNase Enzyme Mix. Pipet up and down to fully mix.
15. Incubate the tube at 22°C for **exactly** 15 minutes 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. Pipet up and down to fully mix.

1.2 RIPA Cell Lysis

Follow the steps below for RIPA Cell Lysis:

1. Add to the sample tube 89.5 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	60.5 µL	66.6 µL	x	8	=	532.8 µL
● 10X RIPA	20 µL	22 µL	x	8	=	176 µL
25X Protease Inhibitors	8 µL	8.8 µL	x	8	=	70.4 µL
● 20% SDS	1 µL	1.1 µL	x	8	=	8.8 µL
Total	**89.5 µL**					

2. Vortex the tube to mix three times for 5 seconds each.
3. Rotate the tube at room temperature for 15 minutes.
4. Spin the tube at 16,000 x g for 2 minutes.
5. Transfer the **SUPERNATANT (clarified lysate)** to a new 1.5 mL tube. This lysate contains the digested chromatin.
6. Continue to Stage 2: Lysate QC, page 16.

Option B: If starting the assay with 10 x 10^6 cells

1.1 Crosslinking and Digestion

NOTES

- 10 x 10^6 cells are needed per HiChIP reaction.
- Pre-freezing the cells is required to get an optimal digestion profile.
- The cell pellet should be frozen at –80°C for at least 30 minutes (step 4). You can keep the cells frozen at –80°C for longer as long as they are not subjected to freeze-thaw.
- All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.

Follow the steps below for Crosslinking and Digestion:

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 1,000 x g for 5 minutes. Carefully discard the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.
5. Thaw your cell pellet **quickly** 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 of 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 discard 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 1X Wash Buffer using a 200 µL pipette 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 discard 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 Before You Begin, page 11).
14. Add 1 µL of ● MNase Enzyme Mix. Pipet up and down to fully mix.
15. Incubate the tube at 22°C for **exactly** 15 minutes 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. Pipet up and down to fully mix.

1.2 RIPA Cell Lysis

Follow the steps below for RIPA Cell Lysis:

1. Add to the sample tube 89 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	60 µL	66 µL	x	8	=	528 µL
● 10X RIPA	20 µL	22 µL	x	8	=	176 µL
25X Protease Inhibitors	8 µL	8.8 µL	x	8	=	70.4 µL
● 20% SDS	1 µL	1.1 µL	x	8	=	8.8 µL
Total	**89 µL**					

2. Vortex the tube to mix three times for 5 seconds each.
3. Rotate the tube at room temperature for 15 minutes.
4. Spin the tube at 16,000 x g for 2 minutes.
5. Transfer the **SUPERNATANT (clarified lysate)** to a new 1.5 mL tube. This lysate contains the digested chromatin.
6. Continue to Stage 2: Lysate QC, page 16.

Stage 2: Lysate QC

As you prepare for Stage 2, keep the following in mind:

- The Lysate QC stage takes ~ 2 hours.
- This stage has 2 objectives:
  - Quantify the clarified lysate.
  - Confirm that the chromatin was properly digested.
- 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 the recommended kits for each system.

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

Figure 2. Stage 2: Lysate QC

Before You Begin

- Verify before use that 100% ethanol was added to the DNA Wash Buffer supplied in the Zymo Research DNA Clean & Concentrator™-5 Kit, as directed by the manufacturer.
- Program the thermal mixer as follows:

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

» ● 10X Crosslink Reversal Buffer might have precipitated in storage. Incubate at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.

Follow the steps below for Lysate QC:

1. Transfer X µL of the clarified lysate (see table below) to a new 1.5 mL tube labeled QC.

Cell Input Amount	Clarified Lysate Volume (X) To Use For QC
1 x 10^6 cells	10 µL
5 x 10^6 cells	2.5 µL
10 x 10^6 cells	2.5 µL

NOTE Store the remainder of the lysate on ice. This is the lysate you will be using in Stage 3. If you are not going to proceed with Stage 3 on the same day, store the remainder of the lysate at -80°C.

2. Add to the QC tube 51.5 µL of a master mix containing the following reagents **in the order listed:**

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	45 µL	49.5 µL	x	8	=	396 µL
● 10X Crosslink Reversal Buffer	5 µL	5.5 µL	x	8	=	44 µL
● Proteinase K	1.5 µL	1.7 µL	x	8	=	13.6 µL
Total	**51.5 µL**					

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

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

4. Purify the QC sample using Zymo Research DNA Clean and Concentrator™-5 Kit (DCC™). Quick spin your QC tube, add 200 µL of DCC™ DNA Binding Buffer, and 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 DCC™ DNA Wash Buffer (see Before You Begin, page 16).
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 DCC™ 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. The 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. As a reference, we typically recover approximately 250 ng and 1,500 ng from 1 x 10^6 and 5 x 10^6 GM12878 cells, respectively and 10,000 ng from 10 x 10^6 iPSCs.
    - Based on the Qubit concentration, your total clarified lysate amount (ng) can be calculated as follows:

      If starting with 1 x 10^6 cell input:

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

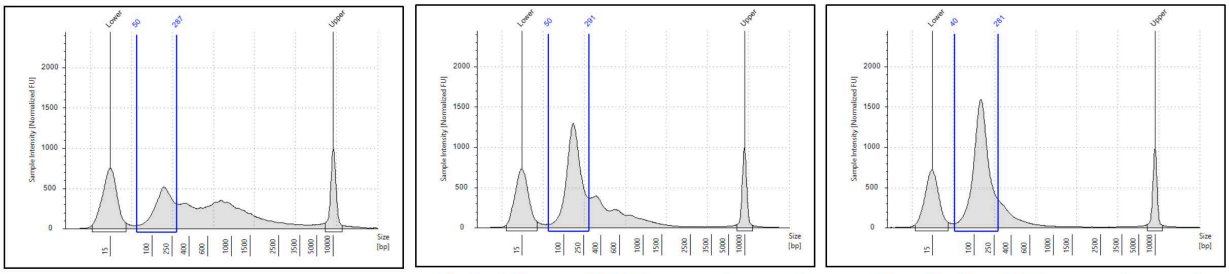
      If starting with 5 x 10^6 or 10 x 10^6 cell input:

      - Total Clarified Lysate (ng) = Qubit reading ng/µL x 10 µL (elution volume) x 80 (dilution factor)

> **NOTE** If the clarified lysate yield is less than the recommended input for HiChIP with your antibody of interest (see Things to Consider Before You Start, page 9), there are 2 options for how to proceed:
>
> **Option 1:** After completing Stage 2 and confirming that the chromatin is optimally digested, freeze the lysate at -80°C. To reach the recommended clarified lysate amount, prepare additional lysate (run Stage 1 with a new cell aliquot), assess the digestion profile (Stage 2), and combine the lysates that are optimally digested before incubating with the antibody (Stage 3).
> **Option 2:** If you still wish to proceed with less than the recommended lysate amount, please note that the library complexity will be reduced (i.e. the library will have a high duplication rate) and you may get poor ChIP enrichment.

14. Check the fragment size distribution of your purified QC sample on a TapeStation HS D5000 ScreenTape. **Make sure your sample is diluted to 1 ng/µL to avoid overloading the tape which results in a misrepresentation of the fragment size distribution.**
    - The digestion profile should contain 40 - 70% mononucleosomes: the first DNA peak, typically in the size range of 50 - 250 bp for the TapeStation, should account for 40 - 70% of total DNA (Figure 3). The size range of the first peak may vary for other analytical instruments such as Bioanalyzer and Fragment Analyzer. If the digestion profile contains 40 - 70% mononucleosomes, proceed to Stage 3: Chromatin Immunoprecipitation, page 20.
    - If the digestion profile contains < 40% mononucleosomes, the chromatin is under-digested (Figure 3). In this case, do not proceed with the rest of the protocol and refer to Appendix 2 to troubleshoot the digestion.
    - If the digestion profile contains > 70% mononucleosomes, the chromatin is over-digested (Figure 3). In this case, do not proceed with the rest of the protocol and refer to Appendix 2 to troubleshoot the digestion.

Figure 3. Different MNase digestion profiles, as analyzed on HS D5000 ScreenTape



Under-digestion (<40% mononucleosomes)

Optimal digestion (40-70% mononucleosomes)

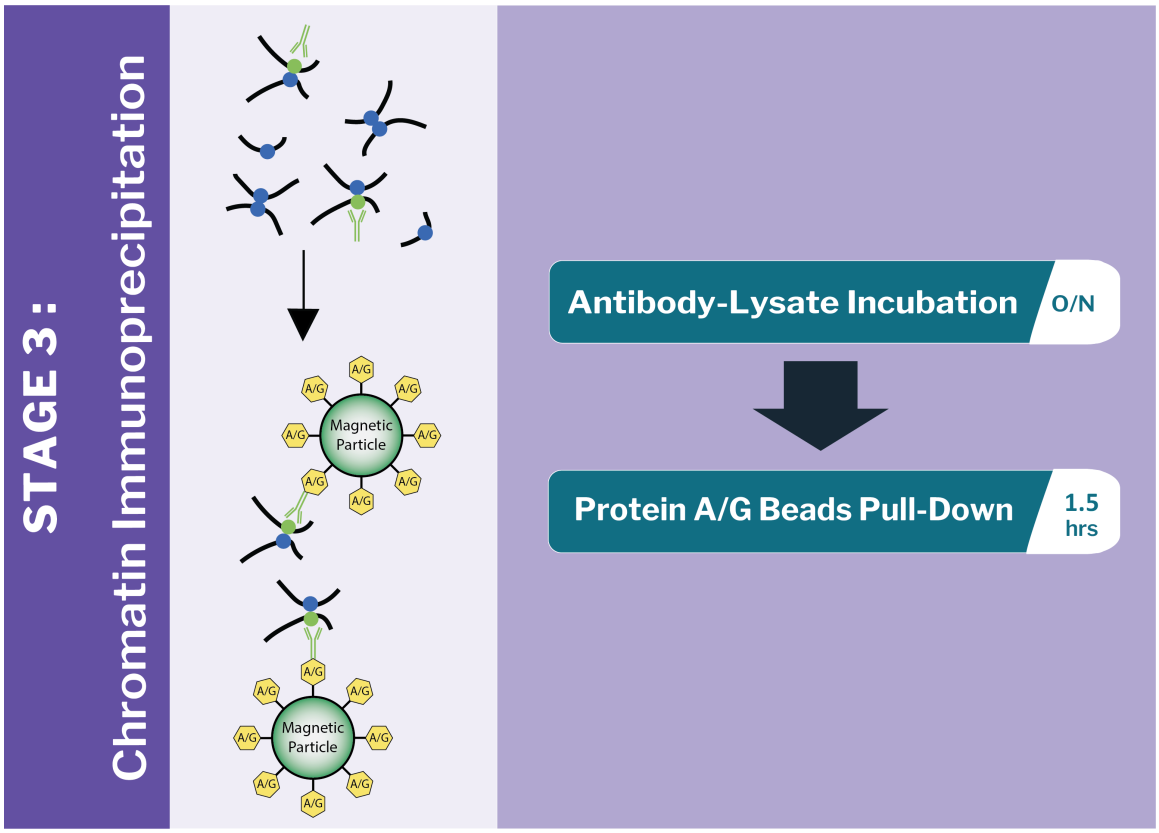
Over-digestion (>70% mononucleosomes)

Stage 3: Chromatin Immunoprecipitation

As you prepare for Stage 3, keep the following in mind:

- Follow best practices when working with beads (see Good Practices, page 8).
- The amount of antibody to add to the lysate is dependent on the antibody of interest.

Figure 4. Stage 3: Chromatin Immunoprecipitation



Before You Begin

- Dilute ● 10X RIPA to 1X with UltraPure Water. Store at room temperature. 1X RIPA is stable at room temperature for 2 months. You need ~4 mL of 1X RIPA per sample.
- Equilibrate Protein A/G beads to room temperature.

3.1 Antibody-Lysate Incubation

Follow the steps below for Antibody-Lysate Incubation:

1. Determine the amount of lysate to use for your antibody of interest (see table below for Dovetail validated antibodies).

Antibody	Supplier	Part Number	Recommended amount (ng or µL) of antibody	Recommended amount (ng) of chromatin (clarified lysate) input
IgG	Cell Signaling	2729	Equivalent amount as the antibody of interest	Equivalent amount as the antibody of interest
CTCF	Cell Signaling	3418	500 ng	1,500
H3K4ac	Active Motif	39381	7.5 µL	1,000
H3K4me3	Cell Signaling	9751	1,250 ng	500
H3K14ac	Cell Signaling	7627	4,000 ng	1,000
H3K27ac	Cell Signaling	8173	300 ng	1,000
H3K27me3	Cell Signaling	9733	1,250 ng	1,000
H3K36me3	Cell Signaling	4909	1,250 ng	1,000
PolII	Abcam	ab26721	20,000 ng	1,500
Klf4	Abcam	ab106629	20,000 ng	10,000
Nanog	Abcam	ab21624	5,000 ng	10,000
Oct4	Cell Signaling	2890	25 µL	10,000
Sox2	Cell Signaling	23064	500 ng	10,000

2. Place the appropriate amount of lysate into a new 1.5 mL tube.

> **NOTES**
>
> - If the amount of lysate is < 150 µL, bring the volume up to at least 150 µL with **1X** RIPA + 25X Protease Inhibitors. This ensures you have sufficient volume for rotation with the antibody. For example, if the lysate amount is 100 µL, bring it up to 150 µL by adding 48 µL of **1X** RIPA and 2 µL of 25X Protease Inhibitors.
> - If multiple cell aliquots of the same sample were processed to reach the required clarified lysate amount, combine the lysates from these preparations at this step prior to incubating with the antibody.

3. Add the recommended amount of antibody (see table above) to the lysate in the 1.5 mL tube.
4. Invert to mix then rotate at 4°C overnight (at least 12 hours).

3.2 Protein A/G Beads Pull-down

Follow the steps below for Protein A/G Beads Pull-down:

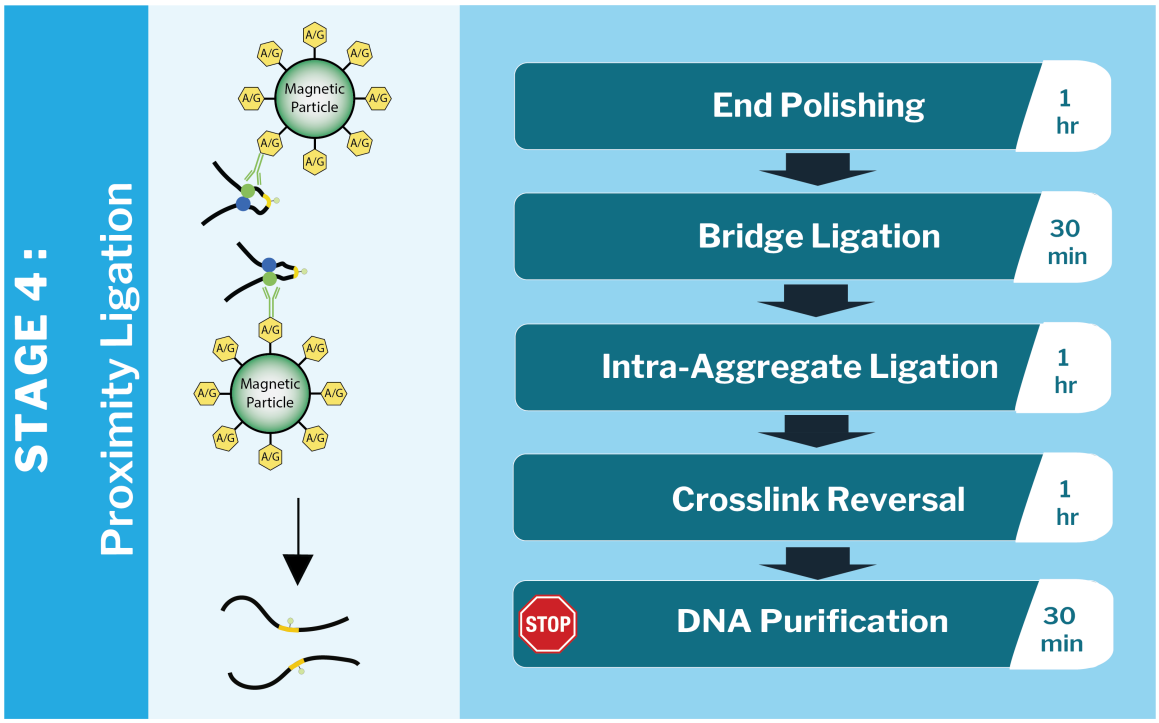
1. Vortex the Protein A/G beads thoroughly (> 30 seconds) to resuspend the beads.
2. Transfer 25 µL of resuspended Protein A/G beads to a new 1.5 mL tube.
3. Place the tube in the magnetic rack for 5 minutes or until the solution looks clear and the beads have fully separated. Discard the supernatant.
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. Discard 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 (see step 4 in Antibody-Lysate Incubation, page 21) and add it to the tube containing the resuspended beads. Pipet up and down to fully mix.
8. Rotate at room temperature for 1 hour.
9. Quick spin the tube and place it in the magnetic rack for 2 minutes. Discard the supernatant.
10. Remove the tube from the magnetic rack and wash the beads with 1 mL 1X RIPA: pipet up and down to resuspend the beads, quick spin and place the tube in the magnetic rack for 2 minutes. Discard the supernatant.
11. Repeat step 10 twice, for a total of 3 washes.
12. Remove the tube from the magnetic rack and wash the beads with 1 mL 1X Wash Buffer: pipet up and down to resuspend the beads, quick spin and place the tube in the magnetic rack for 2 minutes. Discard the supernatant.
13. Repeat step 12 twice, for a total of 3 washes.
14. Continue to Stage 4: Proximity Ligation, page 23. **Do not let the beads dry out.**

Stage 4: Proximity Ligation

As you prepare for Stage 4, keep the following in mind:

- Proximity ligation takes ~ 4 hours.
- Follow best practices when working with beads (see Good Practices, page 8).

Figure 5. Stage 4: Proximity Ligation



Before You Begin

- 10X Crosslink Reversal Buffer might have precipitated in storage. Incubate at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.
- Thaw End Polishing Buffer, 5X Bridge Ligation Buffer, Bridge, and Intra-Aggregate Ligation Buffer at room temperature. Leave on ice once thawed. Vortex to mix prior to use.
- Prepare fresh 80% ethanol for DNA purification with SPRIselect beads for optimal results. Fresh preparations of 80% ethanol will also be used in the remaining stages, 5 and 6. You need a minimum of 1.5 mL for all these stages.
- Equilibrate TE Buffer pH 8.0 to room temperature.

4.1 End Polishing

Follow the steps below for End Polishing:

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

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
● End Polishing Buffer	50 µL	55 µL	x	8	=	440 µL
● End Polishing Enzyme Mix	3.5 µL	3.9 µL	x	8	=	31.2 µL
Total	**53.5 µL**					

2. Pipet up and down to fully mix then transfer the sample to a new 1.5 mL tube.
3. Incubate in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
22°C	30 minutes
65°C	30 minutes

4. Allow the tube to reach room temperature then place it in the magnetic rack for 1 minute (or until the solution looks clear). Discard 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. **Do not remove and discard the supernatant at this step yet**. Keep the tube in the magnetic rack and the beads in buffer to ensure they do not dry out while you prepare for the next reaction.

4.2 Bridge Ligation

Follow the steps below for Bridge Ligation:

1. Prepare and place on ice **fresh** 50 µL Bridge Ligation Mix by mixing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	35 µL	38.5 µL	x	8	=	308 µL
● 5X Bridge Ligation Buffer	10 µL	11 µL	x	8	=	88 µL
● Bridge	5 µL	5.5 µL	x	8	=	44 µL
Total	**50 µL**					

2. Aspirate and discard the supernatant from step 5 in 4.1 End Polishing, page 24. Remove the tube from the magnetic rack and add to the beads:

Reagent	Volume Per Reaction
Bridge Ligation Mix	50 µL
● Bridge Ligase	1 µL
Total	**51 µL**

3. Pipet up and down to fully mix. Incubate at 22°C for 30 minutes 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). Discard 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 discard the supernatant.

4.3 Intra-Aggregate Ligation

Follow the steps below for 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:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
● Intra-Aggregate Ligation Buffer	50 µL	55 µL	x	8	=	440 µL
● Intra-Aggregate Ligation Enzyme Mix	2 µL	2.2 µL	x	8	=	17.6 µL
Total	**52 µL**					

2. Pipet up and down to fully mix. Incubate at 22°C for 1 hour in an agitating thermal mixer set at 1,250 rpm.

> **SAFE STOP** 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). Discard the supernatant.

4.4 Crosslink Reversal

Follow the steps below for 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 **in the order listed:**

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	45 µL	49.5 µL	x	8	=	396 µL
● 10X Crosslink Reversal Buffer	5 µL	5.5 µL	x	8	=	44 µL
● Proteinase K	1.5 µL	1.65 µL	x	8	=	13.2 µL
Total	**51.5 µL**					

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 minutes
68°C	45 minutes
25°C	Hold

> **SAFE STOP** 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.

4.5 DNA Purification

Follow the steps below for DNA Purification on SPRIselect Beads:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 90 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.
3. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
4. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
5. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
6. Leave the tube in the magnetic rack and wash the beads **twice** with 200 µL **fresh** 80% ethanol. Do not resuspend the beads for these washes. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
7. After the last wash, quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol
8. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
9. Off the magnetic rack, resuspend the beads in 57 µL TE Buffer pH 8.0.
10. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
11. Incubate at room temperature, off the magnetic rack, for 5 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. Transfer 55 µL of the **SUPERNATANT** (purified DNA) to a new tube. Discard the beads.

14. Quantify 5 µL of your purified DNA using a Qubit Fluorometer and Qubit dsDNA HS Kit. The amount of DNA recovered is dependent on the antibody used, and, in some cases, may be below the detection limit.

> **NOTE** The amount 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 also be referred to as DNA input.

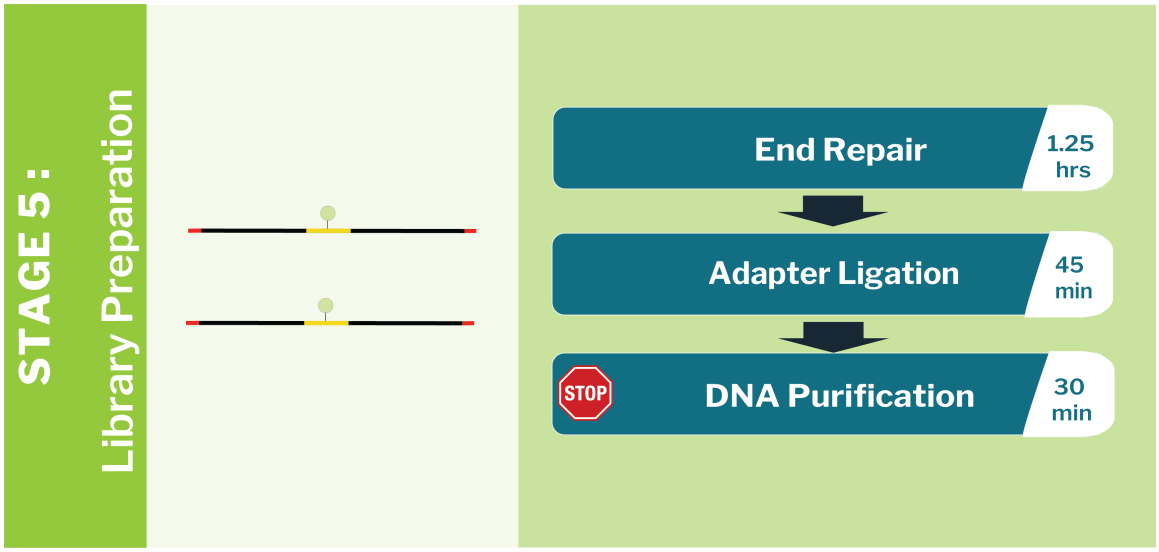
SAFE STOP Purified DNA sample can be stored at -20°C for up to 6 months.

Stage 5: Library Preparation

As you prepare for Stage 5, keep the following in mind:

- The library preparation protocol does not require fragmentation.
- The library preparation protocol takes ~ 2.5 hours.
- Follow best practices when working with beads (see Good Practices, page 8).

Figure 6. Stage 5: Library Preparation



Before You Begin

- The ● End Repair Buffer may have precipitated in storage. Incubate for at least 10 minutes at 37˚C until there is no visible precipitate.
- Equilibrate TE Buffer pH 8.0 to room temperature.
- Thaw ● 250 mM DTT and ● Adaptor for Illumina at room temperature. Vortex to mix prior to use.

5.1 End Repair

Follow the steps below for End Repair:

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:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
End Repair Buffer	7 µL	7.7 µL	x	8	=	61.6 µL
End Repair Enzyme Mix	3 µL	3.3 µL	x	8	=	26.4 µL
250 mM DTT	0.5 µL	0.6 µL	x	8	=	4.8 µL
Total	**10.5 µL**					

3. Pipet up and down to fully mix. Quick spin the tube.
4. Place in a thermal cycler, with the heated lid set to ≥ 75°C, and run the following program:

Temperature	Time
20°C	30 minutes
65°C	30 minutes
12°C	Hold

5.2 Adaptor Ligation and USER Digest

Follow the steps below for Adaptor Ligation and 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 PCR tube containing the end-repaired sample the following reagents:

Reagent	Volume Per Reaction
● Adaptor for Illumina (or diluted Adaptor if DNA input < 5 ng)	2.5 µL
● Ligation Enzyme Mix	30 µL
● Ligation Enhancer	1 µL
Total	**33.5 µL**

> **NOTE** The Ligation Enzyme Mix and Ligation Enhancer can be mixed ahead of time. The master mix is stable for 8 hours at 4˚C. We do not recommend adding the Adaptor for Illumina to the master mix.

3. Pipet up and down to fully mix. Quick spin the tube.
4. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off. Hold at 12°C.
5. Following incubation, add 3 µL of ● USER Enzyme Mix to the PCR tube.
6. Pipet up and down to fully mix. Quick spin the tube.
7. Incubate at 37˚C for 15 minutes in a thermal cycler with the heated lid set to ≥ 47°C. Hold at 12˚C.

5.3 DNA Purification

Follow the steps below for DNA Purification:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 80 µL of resuspended SPRIselect beads to the PCR tube containing the adaptor-ligated sample.
3. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
4. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
5. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
6. Leave the tube in the magnetic rack and wash the beads **twice** with 200 µL **fresh** 80% ethanol. Do not resuspend the beads for these washes. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
7. Quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol
8. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
9. Off the magnetic rack, resuspend the beads in 100 µL TE Buffer pH 8.0.
10. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
11. Incubate at room temperature, off the magnetic rack, for 5 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. Transfer 95 µL of the **SUPERNATANT** (purified adaptor-ligated DNA) to a new tube. Discard the beads.

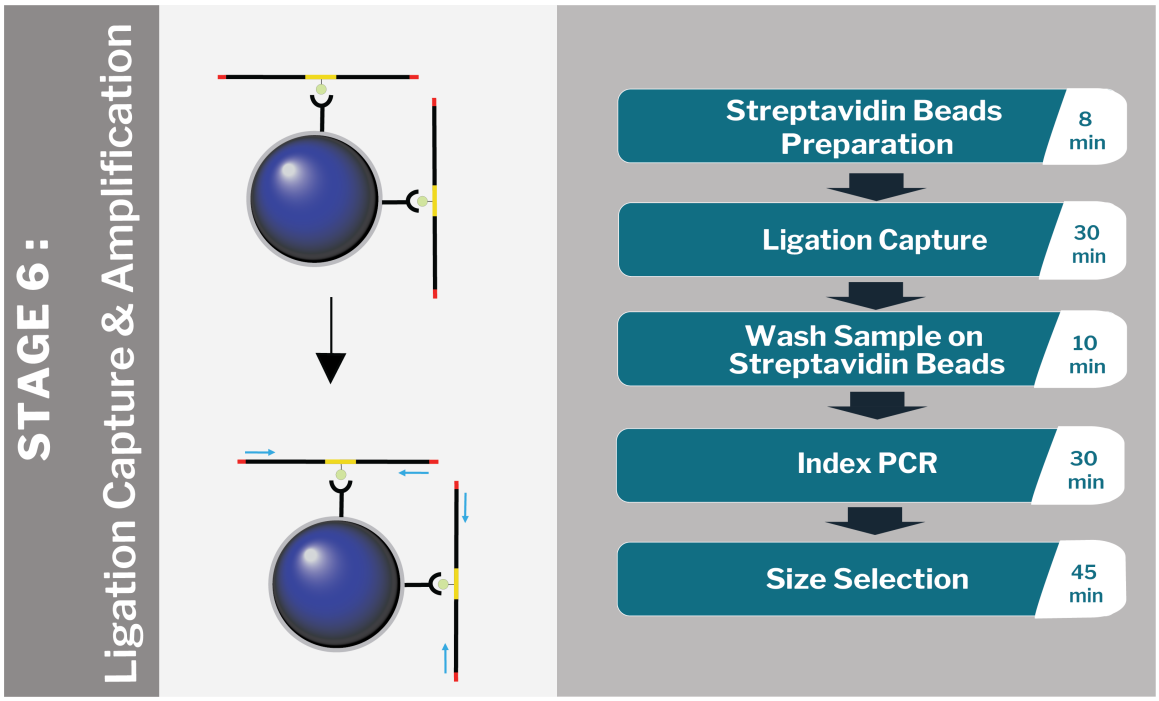
SAFE STOP Purified DNA sample can be stored at -20°C overnight.

Stage 6: Ligation Capture and Amplification

As you prepare for Stage 6, keep the following in mind:

- The Ligation Capture and Amplification protocol takes ~ 2 hours.
- Follow best practices when working with beads (see Good Practices, page 8).

Figure 7. Stage 6: Ligation Capture and Amplification



Before You Begin

- Thaw ● UDI Primer Pair and HotStart PCR Ready Mix and **keep on ice while in use**. Vortex to mix prior to use.
- Equilibrate TE Buffer pH 8.0, ● Streptavidin Beads, ■ TWB, ■ 2X NTB, ■ LWB, and ■ NWB to room temperature.

6.1 Streptavidin Beads Preparation

NOTE This step does not involve any DNA sample.

Follow the steps below for Ligation Capture and Amplification:

1. Vortex the ● Streptavidin Beads vial thoroughly (> 30 seconds) to resuspend the beads. Transfer 25 µL of resuspended ● Streptavidin beads to a new 1.5 mL tube.
2. Place the 1.5 mL tube containing the beads in the magnetic rack for 5 minutes. Discard the supernatant.
3. Remove the tube from the magnetic rack and wash the beads with 200 µL ▬ TWB: pipet up and down to resuspend the beads and place the tube in the magnetic rack for 1 minute. Discard the supernatant.
4. Repeat step 3 once, for a total of 2 washes.
5. After the second wash, resuspend the beads in 100 µL ▬ 2X NTB. Pipet up and down to fully mix.

6.2 Ligation Capture

Follow the steps below for Ligation Capture:

1. Transfer the 95 µL of purified adaptor-ligated DNA (from step 13 in 5.3 DNA Purification, page 31) to the 1.5 mL tube containing the Streptavidin beads resuspended in 100 µL of 2X NTB.
2. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
3. Incubate at 25˚C for 30 minutes in an agitating thermal mixer set at 1,250 rpm.

6.3 Wash Sample on Streptavidin Beads

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 to resuspend the beads, place the tube in the magnetic rack for 1 minute, and discard the supernatant. Remove all of the supernatant between each wash; residual supernatant can interfere with the downstream PCR.

Follow the steps below for Wash Sample on Streptavidin Beads:

1. Quick spin the tube and place it in the magnetic rack for 1 minute. Discard the supernatant.
2. Wash the beads once with 200 µL ▬ LWB.
3. Wash the beads **twice** with 200 µL ▬ NWB.
4. Wash the beads **twice** with 200 µL 1X Wash Buffer.

6.4 Index PCR

> **NOTE** Not all PCR enzymes and master mixes are compatible for amplification in the presence of Streptavidin beads. Please use the HotStart PCR Ready Mix supplied in your Dovetail Kit (Box 2).

Follow the steps below for Index PCR:

1. After the last wash, remove the tube from the magnetic rack and add to the beads 45 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	20 µL	22 µL	x	8	=	176 µL
HotStart PCR Ready Mix	25 µL	27.5 µL	x	8	=	220 µL
Total	**45 µL**					

2. Add 5 µL ● UDI Primer Pair to the PCR reaction. Use one UDI Primer Pair per PCR reaction (see **Appendix 3: Dual Index Primers, page 41**).
3. Pipet up and down to fully mix then transfer to a new 0.2 mL PCR tube.
4. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle). Place the tube into the thermal cycler and run the following program:

Step	Temperature	Time	Cycles
Enzyme Activation	98°C	3 minutes	1
Denature	98°C	20 seconds	See table below
Anneal	65°C	20 seconds	
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	1

DNA Input (Stage 4.5, Step 14)	Cycles
≥ 100 ng	12
< 100 ng - 5 ng	14
< 5 ng	16
undetected	18

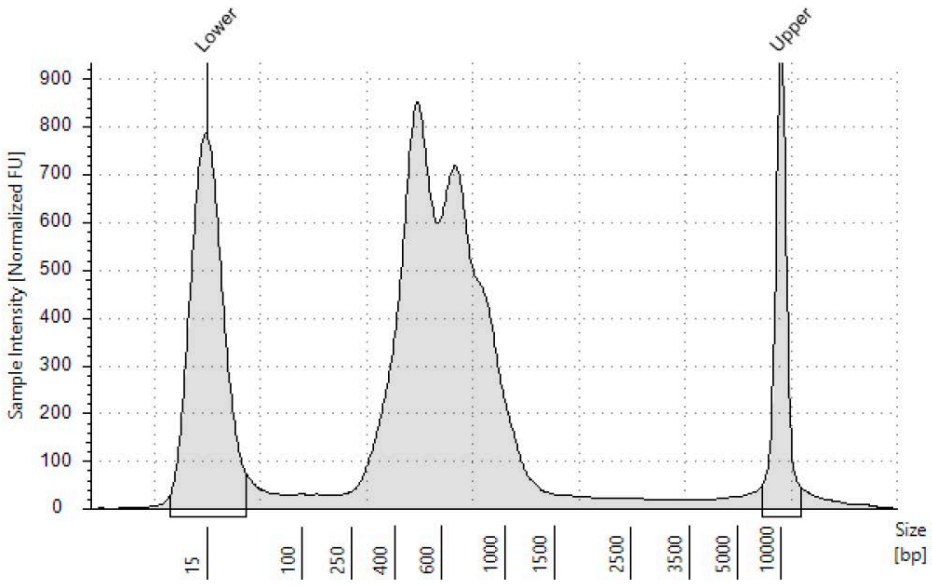
6.5 Size Selection

Follow the steps below for 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. Add 53 µL of TE Buffer pH 8.0 to the 1.5 mL tube to bring the volume of the sample in the tube to 100 µL.
4. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
5. Add 50 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.
6. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
7. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
8. Quick spin the tube and place it in the magnetic rack for 5 minutes.
9. Transfer 145 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.
10. Add 30 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.
11. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
12. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
13. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
14. Leave the tube in the magnetic rack and wash the beads **twice** with 200 µL **fresh** 80% ethanol. Do not resuspend the beads for these washes. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
15. Quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol.
16. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
17. Off the magnetic rack, resuspend the beads in 30 µL TE Buffer pH 8.0.
18. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
19. Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
20. Quick spin the tube and place it in the magnetic rack for 1 minute.
21. Transfer 28 µL of the **SUPERNATANT** to a new 1.5 mL tube. The supernatant is your size selected library. Discard the beads.
22. Quantify your size selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit.
23. 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 (Figure 8).

SAFE STOP The library can be stored at -20°C for up to 6 months.

Figure 8. Expected final library profile, as analyzed on HS D5000 ScreenTape.



Sequencing & QC Analysis

Dovetail® HiChIP *MNase* libraries are sequenced via Illumina® sequencers in paired-end mode. Each library can be sequenced to ~150 M read pairs. We recommend to shallow sequence the library to run a QC analysis prior to deep sequencing. The QC analysis requires ~20 M read pairs (2 x 75 bp, 2 x 100 bp, or 2 x 150 bp). Cantata Bio provides all kit users access to a QC analysis workflow available on readthedocs: https://hichip.readthedocs.io/en/latest/.

Appendix 1: Sample Preparation for 1 x 10^6 Cell Input

As you prepare for 1 x 10^6 cell input, keep the following in mind:

- Sample preparation takes 1.5 hours.

Before You Begin

- The 10X Wash Buffer and ● 20% SDS might have precipitated in storage. Incubate these 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. 1X Wash Buffer is stable at room temperature for 2 months. You need ~6 mL of 1X Wash Buffer per sample for the entire protocol.
- 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.
- Prepare fresh 1X Nuclease Digest Buffer and store at room temperature. 1X Nuclease Digest Buffer is stable for 1 day at room temperature. You need 50 µL of 1X Nuclease Digest Buffer per sample. To prepare 1X Nuclease Digest Buffer (50 µL), mix the following components:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	40 µL	44 µL	x	8	=	352 µL
● 10X Nuclease Digest Buffer	5 µL	5.5 µL	x	8	=	44 µL
● 100 mM $MgCl_2$	5 µL	5.5 µL	x	8	=	44 µL
Total	**50 µL**					

- Set the thermal mixer at 22°C, shaking at 1,250 rpm.
- Thaw ● 0.5 M EGTA at room temperature. Vortex to mix prior to use.

Crosslinking and Digestion

NOTES

- 1 x 10^6 cells are needed per HiChIP reaction.
- Pre-freezing the cells is required to get an optimal digestion profile.
- The cell pellet should be frozen at –80°C for at least 30 minutes (step 4). You can keep the cells frozen at –80°C for longer as long as they are not subjected to freeze-thaw.
- All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.

Follow the steps below for Crosslinking and Digestion:

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 1,000 x g for 5 minutes. Carefully discard the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.
5. Thaw your cell pellet **quickly** 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 of 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 discard 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 discard 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 Before You Begin, page 37).
14. Add 0.5 µL of 1:2 **diluted** MNase Enzyme Mix and pipet up and down to fully mix. To prepare a 1:2 dilution of MNase Enzyme Mix, thoroughly mix 0.5 µL of ● MNase Enzyme Mix and 0.5 µL of 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin, page 37).
15. Incubate the tube at 22°C for **exactly** 15 minutes 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. Pipet up and down to fully mix.

RIPA Cell Lysis

Follow the steps below for RIPA Cell Lysis:

1. Add to the sample tube 144.5 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	115.5 µL	127.1 µL	x	8	=	1,016.8 µL
10X RIPA	20 µL	22 µL	x	8	=	176 µL
25X Protease Inhibitors	8 µL	8.8 µL	x	8	=	70.4 µL
20% SDS	1 µL	1.1 µL	x	8	=	8.8 µL
Total	**144.5 µL**					

2. Vortex the tube to mix three times for 5 seconds each.
3. Rotate the tube at room temperature for 15 minutes.
4. Spin the tube at 16,000 x g for 2 minutes.
5. Transfer the **SUPERNATANT (clarified lysate)** to a new 1.5 mL tube. This lysate contains the digested chromatin.
6. Continue to Stage 2: Lysate QC, page 16.

Appendix 2: Troubleshooting Guide

This Appendix provides guidance for how to troubleshoot MNase digestion that did not pass QC in Stage 2, i.e. the % of mononucleosomes was not between 40 - 70%.

Scenario 1: Chromatin is under-digested with less than 40% mononucleosomes

Solution: Restart Stage 1: Sample Preparation (Crosslinking, Digestion and Lysis), increasing the amount of MNase Enzyme Mix added in step 14. How much to increase the amount of MNase depends on the % of mononucleosomes obtained (i.e. how under-digested was the chromatin?).

% of mononucleosomes between 20 – 40%	% of mononucleosomes < 20%
Increase the amount of MNase added in step 14 by 2-fold	Increase the amount of MNase added in step 14 by 4-fold

Scenario 2: Chromatin is over-digested with more than 70% mononucleosomes

Solution: Restart Stage 1: Sample Preparation (Crosslinking, Digestion and Lysis), decreasing the amount of MNase Enzyme Mix added in step 14. How much to decrease the amount of MNase depends on the % of mononucleosomes obtained (i.e. how over-digested was the chromatin?).

% of mononucleosomes between 70 – 80%	% of mononucleosomes > 80%
You can continue to Stage 3 with caution. The library will have a reduced % of long-range interactions. Alternatively, decrease the amount of MNase added in step 14 by 2-fold	When the chromatin is overdigested with > 80% mononucleosomes, it is difficult to determine the first peak region and accurately quantify the % of mononucleosomes. You can decrease the amount of MNase added in step 14 by 4-fold or contact **support@cantatabio.com** for advice on how to adjust the digestion.

Appendix 3: Dual Index Primers

Dovetail® Dual Index Primer Set #1 for Illumina® includes the following eight UDI Primer Pairs:

Table 7. UDI Primer Pairs

Primer Name	HiSeq® 2000/2500, MiSeq®, NovaSeq® systems i5 index	HiSeq® 3000, 4000, X, NextSeq®, MiniSeq®, iSeq® systems i5 index	All systems i7 index
UDI Primer Pair 1	ATATGCGC	GCGCATAT	CTGATCGT
UDI Primer Pair 2	TGGTACAG	CTGTACCA	ACTCTCGA
UDI Primer Pair 3	AACCGTTC	GAACGGTT	TGAGCTAG
UDI Primer Pair 4	TAACCGGT	ACCGGTTA	GAGACGAT
UDI Primer Pair 5	GAACATCG	CGATGTTC	CTTGTCGA
UDI Primer Pair 6	CCTTGTAG	CTACAAGG	TTCCAAGG
UDI Primer Pair 7	TCAGGCTT	AAGCCTGA	CGCATGAT
UDI Primer Pair 8	GTTCTCGT	ACGAGAAC	ACGGAACA

The indexes on the UDI Primer Pairs are color-balanced in sets of four (1-4, 5-8). Indexes within each group of four are fully color balanced and can be pooled for sequencing. You can multiplex less than four samples, but verify color balance before pooling.