Dovetail® Micro-C Kit

USER GUIDE - TISSUE & BLOOD

VERSION 2.1

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Table of Contents

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Dovetail® Micro-C Kit Components and Storage

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

Table 1. Dovetail® Proximity Ligation Core Box 1 (PN DG-REF-001)

Components	Storage
10X Wash Buffer	2°C to 8°C
NWB Solution	
Chromatin Capture Beads	
Streptavidin Beads*	
20% SDS	
Crosslink Reversal Buffer	

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

Components	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	
Bridge	
Intra-Aggregate Ligation Enzyme Mix	
Intra-Aggregate Ligation Buffer	
Proteinase K	
HotStart PCR Ready Mix*	

*An additional vial of HotStart PCR Ready Mix and Streptavidin Beads are supplied in a bag to support a total of 24 libraries. This is to enable customers looking to prepare 3 technical libraries from the same proximity ligation reactions.

Optional Add-on Modules: Components and Storage

Dovetail® Primer Module Set 1

Table 3. Dovetail® Primer Module Set 1 (PN DG-XG-PM01)

Components	Storage
DG i5 Index 1	-30°C to -10°C
DG i5 Index 2	
DG i5 Index 3	
DG i5 Index 4	
DG i7 Index 1	
DG i7 Index 2	
DG i7 Index 3	
DG i7 Index 4	
DG i7 Index 5	
DG i7 Index 6	

Dovetail® Library Module

Table 4. Dovetail® Library Module (PN DG-LM01)

Components	Storage
End Repair Enzyme Buffer	-30°C to -10°C
End Repair Enzyme Mix	
Ligation Buffer	
Ligation Enzyme Mix	
Stubby Adaptor	

Dovetail® Cell Isolation Module

Table 5. Dovetail® Cell Isolation Module (PN 25023)

Components	Storage
Reconstitution Buffer	2°C to 8°C *
Cell Isolation Enzyme Mix	
100 mM $CaCl_2$	

***Once reconstituted, store the Cell Isolation Enzyme Mix at -20°C.**

User Supplied Reagents, Consumables and Equipment

Reagents

Table 6. 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
TE pH 8.0	Thermo Fisher Scientific	AM9849
DSG (Disuccinimidyl Glutarate)	Thermo Fisher Scientific	A35392
DMSO (Dimethyl Sulfoxide, Anhydrous ≥ 99.99%)	Sigma-Aldrich	276855-100ML
SepMate™-15*	Stemcell Technologies	85415
Lymphoprep™*	Stemcell Technologies	07801
10X RBC Lysis Buffer**	Thermo Fisher Scientific	00-4300
CryoStor®***	Biolife Solutions	210373

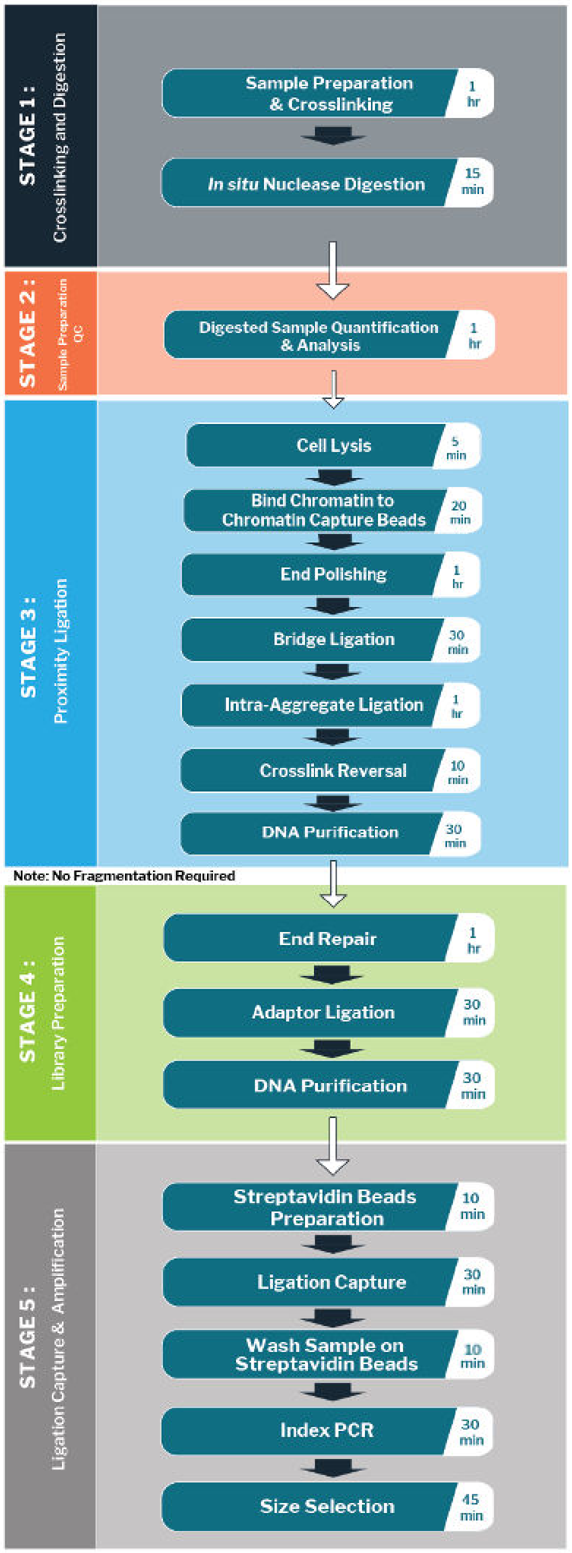
*only needed if working with Fresh Mammalian Whole Blood (≤ 24 hours post collection)
**only needed if working with Fresh Mammalian Whole Blood (24-72 hours post collection)
**** optional for cryopreserving PBMCs

Consumables and Equipment

Table 7. 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		
Swinging Bucket Rotor		
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
MiniStrainer 70 µm mesh	PluriSelect	43-10070-46

Dovetail® Micro-C 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 Chromatin Capture, 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

What is the impact of MNase digestion profiles on conformation analysis?

Achieving an optimal digestion profile which contains 20% - 70% mononucleosomes results in a high complexity library with enriched long-range interactions. When the chromatin is under-digested (< 20% mononucleosomes), the library complexity is lower (i.e. higher duplication rate). In this case, you may need additional libraries to support the sequencing depth needed for your application. When the chromatin is over-digested (>70% mononucleosomes), the library can have a higher fraction of inter-chromosomal information at the expense of cis long-range read pairs.

How much coverage do you need?

The answer to "how much coverage do you need?' is highly dependent on the goal of your experiment. Below are some generalized recommendations.

Topological feature calling	**Resolution**	**Coverage**	**Number of 2 x 150 bp paired-end reads***
A/B compartment	1 Mb	3x	30 million
TADs	50 kb, 25 kb, 10 kb	30x	300 million
Loops	10 kb, 5 kb	80x	800 million

**Assuming mapping to the human genome. These absolute values will vary dependent on species.*

How do you calculate coverage?

The general equation is:
Coverage = [2 x (read length in bp) x (total number of paired-end reads)] / [(genome size in bp)]

Example: For human genomes, 30x coverage is achieved with 300 M paired-end reads of 150 bp.

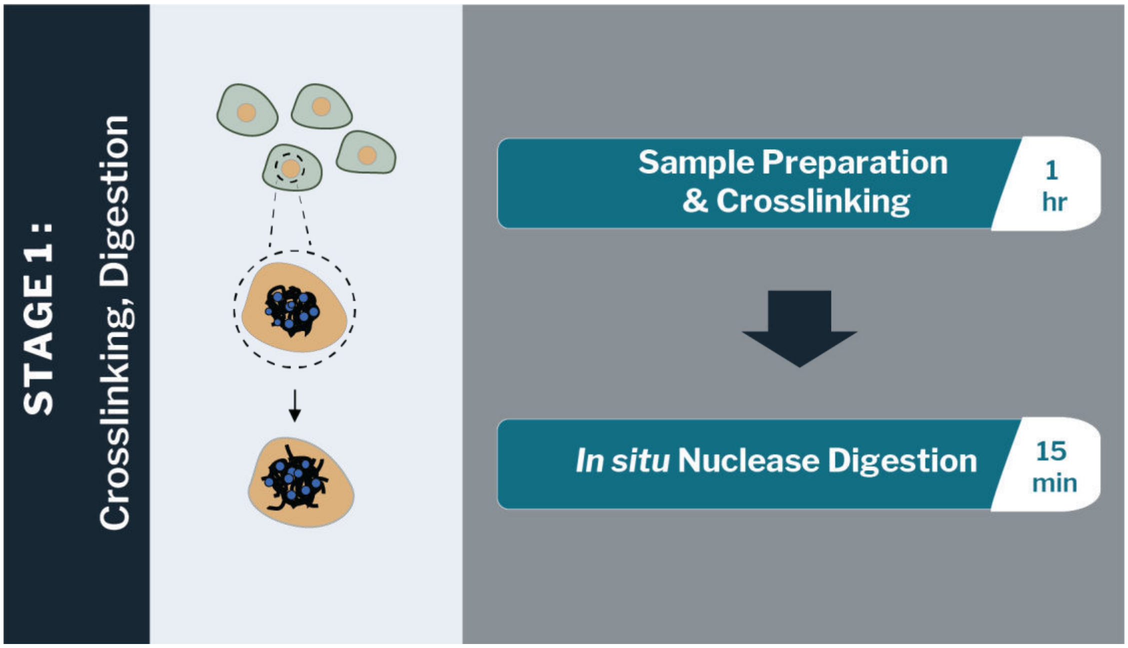
$30x = [\, 2 \times (150 \text{ bp}) \times (300 \times 10^6) \,] \,/\, [\, (3 \times 10^9 \text{ bp}) \,]$

How many libraries are required to achieve the desired coverage?

A Micro-C library can be sequenced up to 300 M read pairs (2 x 150 bp), depending on the library complexity as assessed by the QC analysis of the shallow sequenced library. For human genomes, 30x and 80x coverage can be achieved with one and three Micro-C libraries respectively, assuming the libraries are of high complexity.
This slide deck describes in detail the different approaches one can take to prepare multiple libraries that are needed to support the desired coverage/depth.

Stage 1: Crosslinking and Digestion

Figure 1. Stage 1: Crosslinking and Digestion



A. TISSUE

Before You Begin

- ☐ The 10X Wash Buffer might have precipitated in storage. Incubate this solution at 50°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 ~2 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.
- ☐ Reconstitute Cell Isolation Enzyme Mix as follows: on ice, transfer 850 µL of Reconstitution Buffer to the tube containing Cell Isolation Enzyme Mix powder. Pipet up and down to mix. Transfer an additional 800 µL of Reconstitution Buffer, so the powder is now resuspended in a total of 1,650 µL Buffer. Pipet up and down to mix. Incubate on ice for 30 minutes. Pipet mix again before use. **Reconstituted Cell Isolation Enzyme Mix should be stored at -20°C** and is stable for 1 year after reconstitution. Thaw reconstituted Cell Isolation Enzyme Mix on ice as it is temperature sensitive.

☐ 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.

Follow the steps below for Crosslinking and Digestion:

1. Weigh out the appropriate mass of frozen tissue based on the table below:

Tissue	Input Mass
Liver	5 mg
Brain	5 mg
Lung	5 mg
Heart	5 mg
Spleen	3 mg
Muscle	20 mg

2. Grind the tissue to a **fine powder** with a mortar and pestle in a **liquid nitrogen bath**.
3. Transfer the ground tissue to a 1.5 mL tube.

NOTES

- You may choose to freeze your ground tissue pellet at -80°C at this stage. Otherwise, proceed with the protocol.
- All crosslinking reactions (steps 5 – 11) should be carried out at room temperature.

4. Resuspend the tissue pellet in 200 µL of 1X PBS and pipet up and down to ensure no clumps are present.
5. Add 2 µL of 0.3 M DSG.
6. Rotate the tube for **30 minutes** at room temperature. Sample should not settle.
7. Add 5.4 µL of 37% formaldehyde.
8. Rotate the tube for **30 minutes** at room temperature. Sample should not settle.
9. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette**.
10. Resuspend the pellet in 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

NOTES Ensure that your tissue sample is fully resuspended in solution by rinsing the sides of the tube with Wash Buffer

11. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette**.
12. Resuspend the tissue pellet in:
    - 100 µL of reconstituted Cell Isolation Enzyme Mix (see Before You Begin)
    - 5 µL of 100 mM $CaCl_2$

NOTES After using the reconstituted Cell Isolation Enzyme Mix vial, store it at -20°C.

13. Incubate the tube in a thermal mixer WITHOUT SHAKING at 37°C for 30 minutes.
14. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette**.
15. Resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin).
16. Add 0.5 µL of MNase Enzyme Mix. Pipet up and down to fully mix.
17. 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.
18. Stop the reaction by adding 5 µL of 0.5 M EGTA. Pipet up and down to fully mix.
19. **For muscular tissues (such as muscle or heart)**, filter your sample through the 70 µm MiniStrainer to filter out the cell aggregates. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell/tissue mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter and discarded. You will be proceeding with the rest of the protocol with the filtrate which should be in single-cell suspension in the tube. **If you are not working with muscular tissues, skip this step and proceed to step 20.**
20. Continue to Stage 2: Sample preparation QC.

B. Cryopreserved Peripheral Blood Mononuclear Cells (PBMCs)

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

- Sample preparation takes ~ 1.5 hours.
- It is essential to work quickly and limit handling of the PBMCs once they are thawed.
- At any point in the protocol if there are cell aggregations observed, it is essential to filter out the aggregates using MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

Before You Begin

- ☐ The 10X Wash Buffer might have precipitated in storage. Incubate these solutions at 50°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 ~12 mL of 1X Wash Buffer per sample for the entire protocol. **Warm 10mL of 1X Wash Buffer to 37 °C for 15 minutes in a 15 mL tube.**
- ☐ 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.
- ☐ The cell centrifugation steps must be carried out in a swinging bucket rotor. Using a swinging bucket rotor reduces cell loss.
- ☐ 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.

Follow the steps below for Crosslinking and Digestion:

1. Quickly thaw cryopreserved PBMCs stock in a 37°C water bath for approximately 2-5 minutes.
2. Transfer the thawed PBMC mix to the tube containing the 10 mL of pre-warmed 1X Wash buffer (see Before You Begin). Take care to pipet gently.
3. Spin the cells at 500 x g for 5 minutes in a swinging bucket rotor. Discard the supernatant by pouring it out gently (do not disturb the pellet), pipet out any remaining supernatant.

4. Resuspend the pellet with 200 µL of 1X PBS, pipet up and down gently to break up clumps and resuspend the pellet.

> NOTE If **cell clumping** is observed at this stage, filter the sample through the **MiniStrainer** . Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

5. Take an aliquot to count the cells. Keep the remaining cells on ice until the cells are counted. **Count quickly, this is a delicate sample.**

> NOTES
>
> - 1×10^6 cells are recommended per Micro-C reaction.
> - Remaining cells can be cryopreserved in Cryostor® or DMSO and FBS (see Appendix 3).

6. Gently, pipette mix the PBMCs on ice. Transfer a volume equivalent to 1×10^6 cells to a new 1.5 mL tube.
7. Bring up the volume to 200 µL with 1X PBS.

> NOTE All crosslinking reactions (steps 8 – 14) should be carried out at room temperature.

8. Add 2 µL 0.3 M DSG. Pipet to mix.
9. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
10. Add 5.4 µL of 37% formaldehyde.
11. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
12. Spin the tube at 500 x g in a swinging bucket rotor for 5 minutes. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
13. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
14. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
15. Resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin).
16. Add 0.5 µL of MNase Enzyme Mix. Pipet up and down to fully mix.
17. 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.
18. Stop the reaction by adding 5 µL of 0.5 M EGTA. Pipet up and down to fully mix.
19. Continue to Stage 2: Sample Preparation QC.

C. Fresh Mammalian Whole Blood (≤ 24 hours post collection)

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

- Sample preparation takes ~ 1.5 hours.
- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood ≤ 24 hours post collection.
- Fresh blood should be shipped and/or stored at **ambient temperature**. Blood samples processed within 24 hours of collection yield higher quality and quantity of PBMCs.
- If you are processing a blood sample between 24-72 hours of collection, please refer to protocol D below, as SepMate™ works best on fresh samples. We do not recommend use of blood processed >72 hours post collection.
- At any point in the protocol if there are cell aggregations observed, it is essential to filter out the aggregates using MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.
- SepMate™-15 is designed to process 0.5 to 5 mL of blood samples. Please follow manufactures guidelines for volume recommendation.
- Typically, 0.5 to 3 x 10^6 PBMCs are isolated from 1 mL of healthy whole blood.
- It is essential to work quickly and limit handling of the PBMCs once they are isolated from whole blood.

Before You Begin

- ☐ The 10X Wash Buffer might have precipitated in storage. Incubate these solutions at 50°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 ~4 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.
- ☐ Warm Lymphoprep™ to room temperature (15 - 25 °C) before use.
- ☐ The 1X PBS solution should be at room temperature.
- ☐ The cell centrifugation steps must be carried out at room temperature in a swinging bucket rotor. Using a swinging bucket rotor reduces cell loss.
- ☐ 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.

Follow the steps below for Crosslinking and Digestion:

1. Place a SepMate™ tube in tube rack and keep it vertical.
2. Mix the Lymphoprep™ reagent thoroughly by inverting the bottle several times before use.
3. Add 4.5 mL of Lymphoprep™ to the SepMate™ tube by carefully pipetting it through the center hole of the column insert. The top of the Lymphoprep™ reagent will be above the insert.
4. Mix the blood sample gently by pipetting. Transfer 3 mL of blood to a new 15 mL tube.
5. Dilute the blood with 3 mL of 1X PBS. Mix gently by pipetting up and down.
6. Add the diluted blood sample to the SepMate™ tube by pipetting it down the side of the tube. Take care not to pipette the sample directly through the central hole.
7. Centrifuge at 1,200 x g for 20 minutes at room temperature, with the brake on.
8. Pour the top layer containing the PBMCs into a new 15 mL tube.

> NOTE Do not hold the tube in the inverted position for longer than 2 seconds. Some amount of red blood cells may be present in the transfer. These RBCs will not affect the Micro-C assay.

9. Add equal volume of 1X PBS to dilute the enriched PBMCs. Invert to mix.
10. Centrifuge the PBMC containing tube at 500 x g for 5 minutes. Discard the supernatant.
11. Resuspend the pellet in 200 µL of 1X Wash Buffer, pipet up and down gently to break up clumps and resuspend the pellet.
12. Centrifuge at 500 x g for 5 minutes. Carefully discard the supernatant.
13. Resuspend the pellet with 200 µL of 1X PBS, pipet up and down gently to break up clumps and resuspend the pellet.

NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

14. Take an aliquot to count the cells. Keep the remaining cells on ice until cells are counted. **Count quickly, this is a delicate sample.**

NOTES:

- 1×10^6 cells are recommended per Micro-C reaction.
- Remaining cells can be cryopreserved in CryoStor® or DMSO and FBS (see Appendix 3)

15. Gently, pipette mix the PBMCs on ice. Transfer 1×10^6 cells into a new 1.5 mL tube.
16. Bring up the volume to 200 µL with 1X PBS.

NOTE All crosslinking reactions (steps 17 – 23) should be carried out at room temperature.

17. Add 2 µL of 0.3 M DSG.
18. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
19. Add 5.4 µL of 37% formaldehyde.
20. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
21. Spin the tube at 500 x g in a swinging bucket rotor for 5 minutes. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
22. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
23. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
24. Resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin).
25. Add 0.5 µL of MNase Enzyme Mix. Pipet up and down to fully mix.
26. 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.
27. Stop the reaction by adding 5 µL of 0.5 M EGTA. Pipet up and down to fully mix.
28. Continue to Stage 2: Sample Preparation QC.

D. Fresh Mammalian Whole Blood (24-72 hours post collection)

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

- Sample preparation takes ~ 1.5 hours.
- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood 24 - 72 hours post collection.
- Fresh blood should be shipped and/or stored at **ambient temperature**. Blood samples processed within 24 hours of collection yield higher amount and quantity of PBMCs.
- We do not recommend use of blood processed >72 hours post collection.
- At any point in the protocol if there are cell aggregations observed, it is essential to filter out the aggregates using MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.
- The 10X RBC Lysis Buffer contains ammonium chloride which is optimal for lysis of erythrocytes with minimal effect on lymphocytes.
- Typically, 0.5 to 3 x 10^6 PBMCs are isolated from 1 mL of healthy whole blood.
- It is essential to work quickly and limit handling of the PBMCs once they are isolated from whole blood.

Before You Begin

☐ The 10X Wash Buffer might have precipitated in storage. Incubate these solutions at 50°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 ~4 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.

☐ Warm 10X RBC Lysis Buffer to room temperature (15 - 25 °C) before use.

☐ The 1X PBS solution should be at room temperature.

☐ The cell centrifugation steps must be carried out at room temperature in a swinging bucket rotor. Using a swinging bucket rotor reduces cell loss.

☐ 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.

Follow the steps below for Crosslinking and Digestion:

1. In a 50 mL tube, prepare 30 mL of 1X RBC Lysis Buffer by adding 3mL of 10X RBC lysis buffer to room temperature 27 mL of molecular grade water. Mix by inverting.
2. Mix the blood sample gently by pipetting. Transfer 3 mL of blood to the 50 mL centrifuge tube containing 30 mL of 1X RBC Lysis Buffer. Mix by inverting.
3. Incubate for 10-15 minutes at room temperature (no more than 15 minutes).
4. Spin the cells at 500 x g for 5 minutes. Discard the supernatant.
5. Resuspend the pellet in 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and resuspend the pellet.
6. Centrifuge at 500 x g for 5 minutes. Carefully discard the supernatant.
7. Resuspend the pellet with 200 µL of 1X PBS, pipet up and down gently to break up clumps and resuspend the pellet.

NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

8. Take an aliquot to count the cells. Keep the remaining cells on ice until cells are counted. **Count quickly, this is a delicate sample.**

   NOTES:

   - 1×10^6 cells are recommended per Micro-C reaction.
   - Remaining cells can be cryopreserved in CryoStor® or DMSO and FBS (see Appendix 3)

9. Gently, pipette mix the PBMCs on ice. Transfer 1×10^6 cells to a new 1.5mL tube.
10. Bring up the volume to 200 µL with 1X PBS.

NOTE All crosslinking reactions (steps 11 – 17) should be carried out at room temperature.

11. Add 2 µL of 0.3 M DSG.
12. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
13. Add 5.4 µL of 37% formaldehyde.
14. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
15. Spin the tube at 500 x g in a swinging bucket rotor for 5 minutes. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
16. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
17. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
18. Resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin).
19. Add 0.5 µL of MNase Enzyme Mix. Pipet up and down to fully mix.
20. 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.
21. Stop the reaction by adding 5 µL of 0.5 M EGTA. Pipet up and down to fully mix.
22. Continue to Stage 2: Sample Preparation QC.

Stage 2: Sample Preparation QC

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

- ☐ The Sample Preparation QC stage takes ~ 1 hour.
- ☐ This stage has 2 objectives:
  - ☐ Quantify the total digested sample to determine the sample volume to use in Stage 3.
  - ☐ 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: Sample Preparation QC

Before You Begin

- ☐ 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 3,4, and 5. You need a minimum of 2 mL for all these stages.
- ☐ Program the thermal mixer as follows:

Temperature	Time
78°C	10 minutes
25°C	Hold

- ☐ The Crosslink Reversal Buffer might have precipitated in storage. Incubate at 50°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.

Follow the steps below for Sample Preparation QC:

1. Pipet mix the digested sample from Stage 1, then transfer 2.5 µL to a new 1.5 mL tube labeled QC.

> ☐ NOTE Store the remainder of your digested sample on ice. This is what 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 sample 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
Crosslink Reversal Buffer	50 µL	55 µL	x	8	=	440 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
78°C	10 minutes
25°C	Hold

4. Quick spin the QC tube after incubation.
5. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend. Add 90 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. Discard the supernatant.
9. 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. Add the ethanol, wait for 1 minute then discard the ethanol supernatant.
10. 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.
11. Air dry the beads in the magnetic rack for 5 minutes until no residual ethanol remains. Do not over dry the beads.
12. Off the magnetic rack, resuspend the beads in 10 µL TE Buffer pH 8.0.
13. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
14. Incubate at room temperature, off the magnetic rack, for 5 minutes.
15. Quick spin the tube and place it in the magnetic rack for 1 minute (or until the solution looks clear).
16. Transfer 8 µL of the **SUPERNATANT** (purified DNA) to a new tube. This new tube contains your purified QC DNA. Discard the beads.

17. Quantify the purified QC DNA with a Qubit® Fluorometer and Qubit® dsDNA HS Kit.

    ☐ Based on the Qubit concentration, the total digested sample (ng) can be calculated as follows:
    Total digested sample (ng) = Qubit reading ng/μL x 10 μL (elution volume) x 22.2 (dilution factor)

    ☐ At the start of Stage 3, the digested sample will be lysed by SDS and will be referred to as lysate. "Total lysate" and "total digested sample" are used interchangeably when referring to the yield quantification (i.e. the ng amounts are equal). You will use in Stage 3 a volume of the lysate that corresponds to up to 1,500 ng. This volume can be calculated as follows:

    $$\text{Volume } (\mu L) = \frac{1{,}500 \text{ (ng)} \times 55.5 \ (\mu L)}{\text{Total digested sample (ng)}}$$

    ☐ If the total lysate is < 1,500 ng, use all of the lysate in Stage 3.

    ☐ If the total lysate is > 1,500 ng, store the remainder of the lysate at -80°C.

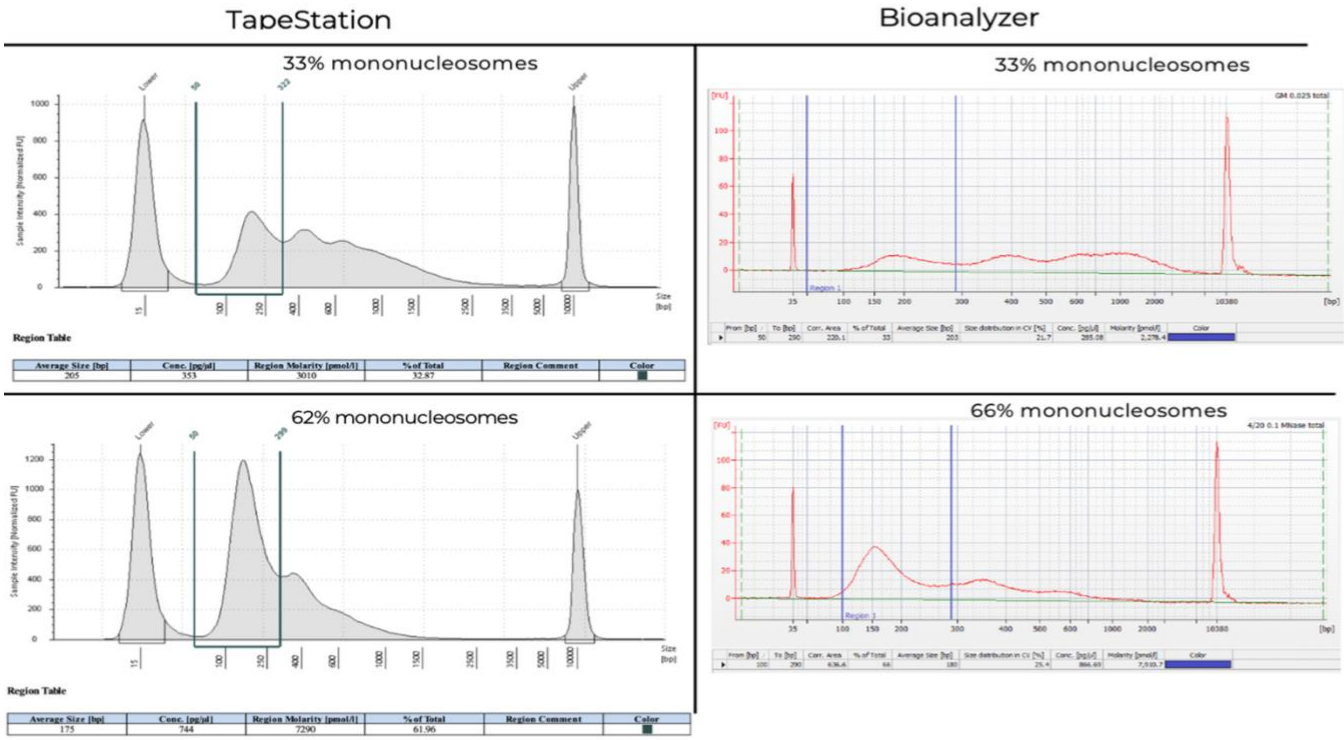
18. Check the fragment size distribution of your purified QC sample on a TapeStation HS D5000 ScreenTape. Make sure your sample is diluted to 1 ng/μL.

    ☐ For optimal nucleosome-level resolution, the digestion profile should contain 20% - 70% mononucleosomes: the first DNA peak, typically in the size range of 50 - 300 bp for TapeStation, should account for 20% - 70% of total DNA (Figure 3). The size range of the peak may vary for other analytical instruments such as Bioanalyzer and Fragment Analyzer. If the digestion profile contains 20% - 70% mononucleosomes, proceed to Stage 3: Proximity Ligation.

    ☐ If the digestion profile contains < 20% mononucleosomes, the library complexity will be lower (i.e. higher duplication rate). In this case, you may need additional libraries to support the sequencing depth needed for your application. You have 2 options: proceed to Stage 3 knowing that additional library(ies) are likely needed **or** redigest your sample before proceeding to Stage 3 (refer to Appendix 2: Troubleshooting Guide for sample redigestion).

    ☐ If the digestion profile contains > 70%, you may proceed to Stage 3: Proximity Ligation with caution. The library may include a higher fraction of inter-chromosomal information at the expense of cis long-range read pairs. This profile is likely due to suboptimal cell counting or a significant cell loss in the washing steps after crosslinking. Alternatively, you can re-start the protocol and decrease the volume of MNase Enzyme Mix in step 13 in Stage 1: Crosslinking and Digestion. For advice on what volume to use, please contact support@cantatabio.com.

Figure 3. Examples of QC Pass digestion profiles, as analyzed on HS D5000 ScreenTape and HS DNA Kit



Region Table

Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
205	353	3010	32.87		■

From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
50	290	220.1	33	203	21.7	285.08	2,278.4	■

Region Table

Average Size [bp]	Conc. [pg/µl]	Region Molarity [pmol/l]	% of Total	Region Comment	Color
175	744	7290	61.96		■

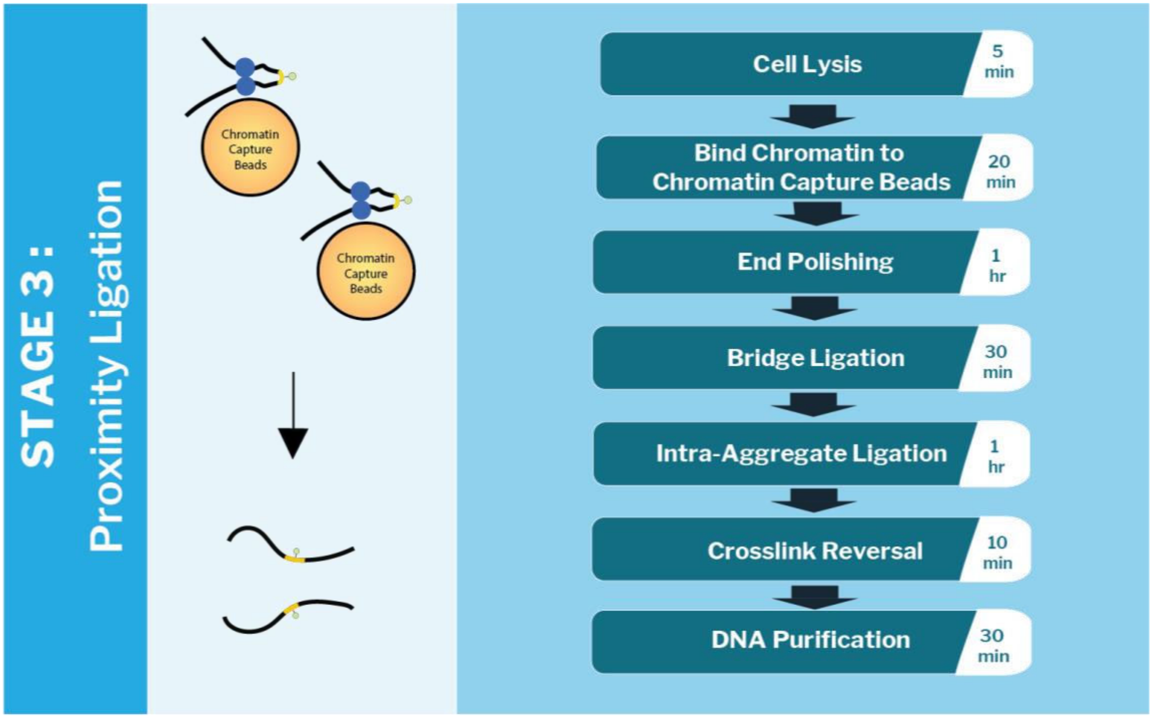
From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distribution in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]	Color
100	290	636.6	66	180	25.4	866.60	7,910.7	■

Stage 3: Proximity Ligation

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

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

Figure 4. Stage 3: Proximity Ligation



Before You Begin

- ☐ The Crosslink Reversal Buffer and 20% SDS might have precipitated in storage. Incubate at 50°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.
- ☐ Equilibrate Chromatin Capture Beads to room temperature.

3.1 Bind Chromatin to Chromatin Capture Beads

Follow the steps below for Bind Chromatin to Chromatin Capture Beads:

1. Thaw your digested sample at room temperature, if stored at -80°C (step 1 NOTE in Stage 2: Sample Preparation QC).
2. Add 3 µL of 20% SDS to the fully thawed digested sample tube. Pipet up and down to fully mix. **This is your lysate**.
3. Incubate the lysate at 22°C for 5 minutes in an agitating thermal mixer set at 1,250 rpm.
4. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
5. Transfer 100 µL of resuspended Chromatin Capture Beads to a new 1.5 mL tube.
6. Pipet mix the lysate. Transfer 1,500 ng of the lysate to the 1.5 mL tube containing the beads. If the total amount is <1,500 ng, add the entire lysate.
7. Pipet up and down to fully mix. Incubate at room temperature, off the magnetic rack, for 10 minutes.
8. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Discard the supernatant.
9. Remove the tube from the magnetic rack and wash the beads 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.
10. Remove the tube from the magnetic rack and resuspend the beads in 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

3.2 End Polishing

Follow the steps below for End Polishing:

1. Prepare 53 µL of End Polishing 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 µL	3.3 µL	x	8	=	26.4 µL
Total	53 µL					

2. Place the tube from step 10 in Stage 3.1 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
3. Remove the tube from the magnetic rack and immediately add to the beads 53 µL of the End Polishing master mix.
4. 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 minutes
65°C	30 minutes

5. Allow the tube to reach room temperature then quick spin and place it in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
6. Remove the tube from the magnetic rack and resuspend the beads in 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction**.

3.3 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. Place the tube from step 6 in Stage 3.2 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
3. Remove the tube from the magnetic rack and immediately add to the beads:

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

4. Pipet up and down to fully mix. Incubate at 22°C for 30 minutes in an agitating thermal mixer set at 1,250 rpm.
5. Quick spin the tube, then place it in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
6. Remove the tube from the magnetic rack and resuspend the beads in 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

3.4 Intra-Aggregate Ligation

Follow the steps below for Intra-Aggregate Ligation:

1. Prepare 52 µL of Intra-Aggregate Ligation 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. Place the tube from step 6 in Stage 3.3 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
3. Remove the tube from the magnetic rack and immediately add to the beads 52 µL of the Intra-Aggregate Ligation master mix.
4. 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.

5. Quick spin the tube, then place it in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.

3.5 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
Crosslink Reversal Buffer	50 µL	55 µL	x	8	=	440 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
78°C	10 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. Quick spin the tube, then place it in the magnetic rack for 1 minute. **Transfer** 50 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.

3.6 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. 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 52 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 50 µL of the **SUPERNATANT** (purified DNA) to a new tube. Discard the beads.
14. Quantify the purified DNA using a Qubit Fluorometer and Qubit dsDNA HS Kit.

    >> If you are using the Micro-C Kit for genome-wide analysis (such as calling A/B compartments, TADs, and loops), you will use 150 ng of your purified DNA per library prep for Stage 4 in a 50 µL volume. If needed, you can bring up the volume to 50 µL using TE Buffer pH 8.0.

    - ☐ If you recovered < 150 ng, use all of the purified DNA to proceed to Stage 4.
    - ☐ If you recovered > 150 ng, use 150 ng to proceed to Stage 4 and keep the remaining purified DNA stored at -20°C. You can use the remaining DNA to prepare additional libraries if your application requires more complexity or coverage (see Things to Consider Before You Start).

    >> If you are using the Micro-C Kit together with Dovetail® Pan Promoter Enrichment Kit to study the chromatin topology anchored at gene promoter sites, we recommend using **up to 1000 ng of the purified DNA and Custom Adaptor** for Stage 4. Increasing the amount of DNA going into library preparation increases the complexity of the **enriched** library (i.e. lowers the duplication rate). Please note, if less than 150 ng of DNA were recovered and used for library preparation, the enriched library will likely be of less than desired complexity.

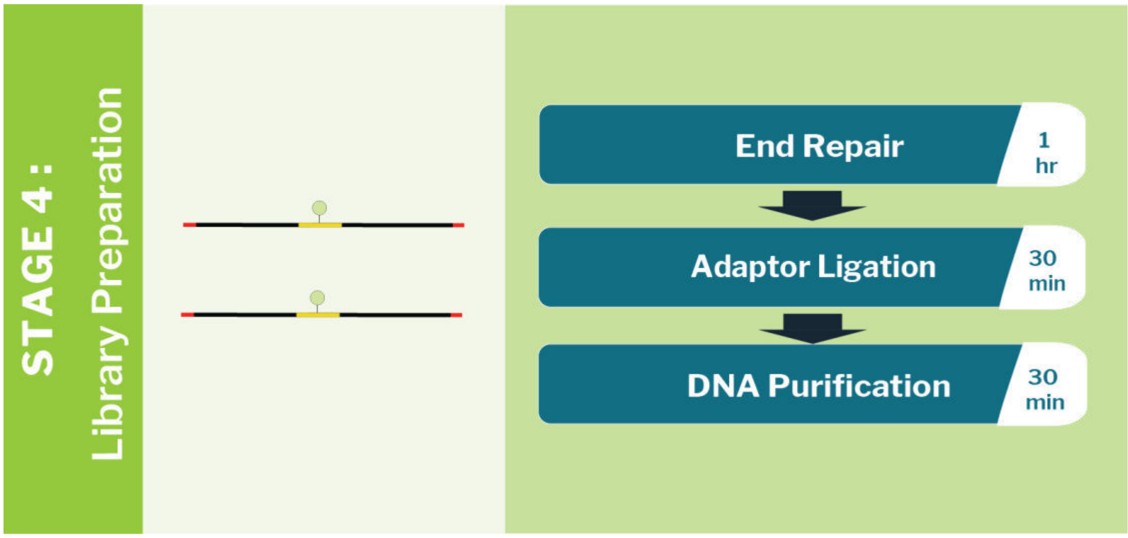
☐ SAFE STOP Purified DNA sample can be stored at -20°C for up to 6 months.

Stage 4: Library Preparation

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

☐ The library preparation protocol does not require fragmentation.
☐ The library preparation protocol takes ~ 2 hours.
☐ Follow best practices when working with beads (see Good Practices).

Figure 5. Stage 4: 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.

☐ Thaw Stubby Adaptor at room temperature. Vortex to mix prior to use.

4.1 End Repair

Follow the steps below for End Repair:

1. To a new 0.2 mL PCR tube, transfer 150 ng of the purified DNA (or up to 1000 ng of the purified DNA if performing Pan Promoter Enrichment) and bring up the volume to 50µL with UltraPure water, if needed.
2. Add to the PCR tube 10 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
Total	10 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

4.2 Adaptor Ligation

Follow the steps below for Adaptor Ligation:

1. Add to the PCR tube containing the end-repaired sample the following reagents in order:

Reagent	Volume Per Reaction
Stubby Adaptor	2.5 µL
Ligation Buffer	25 µL
Ligation Enzyme Mix	5 µL
Total	32.5 µL

☐ NOTE The Ligation Enzyme Mix and Ligation Buffer can be mixed and used as a master mix. We do not recommend adding the Stubby Adaptor to the master mix. If using a master mix, add the Stubby Adaptor first then add 30 µL of the Ligation master mix.

2. Pipet up and down to fully mix. Quick spin the tube.
3. Incubate at 20°C for 15 minutes in a thermal cycler with the heated lid off. Hold at 12°C.

4.3 DNA Purification

Follow the steps below for DNA Purification:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 85 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. 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 22 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 (or until the solution looks clear).
13. **Transfer** 20 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.

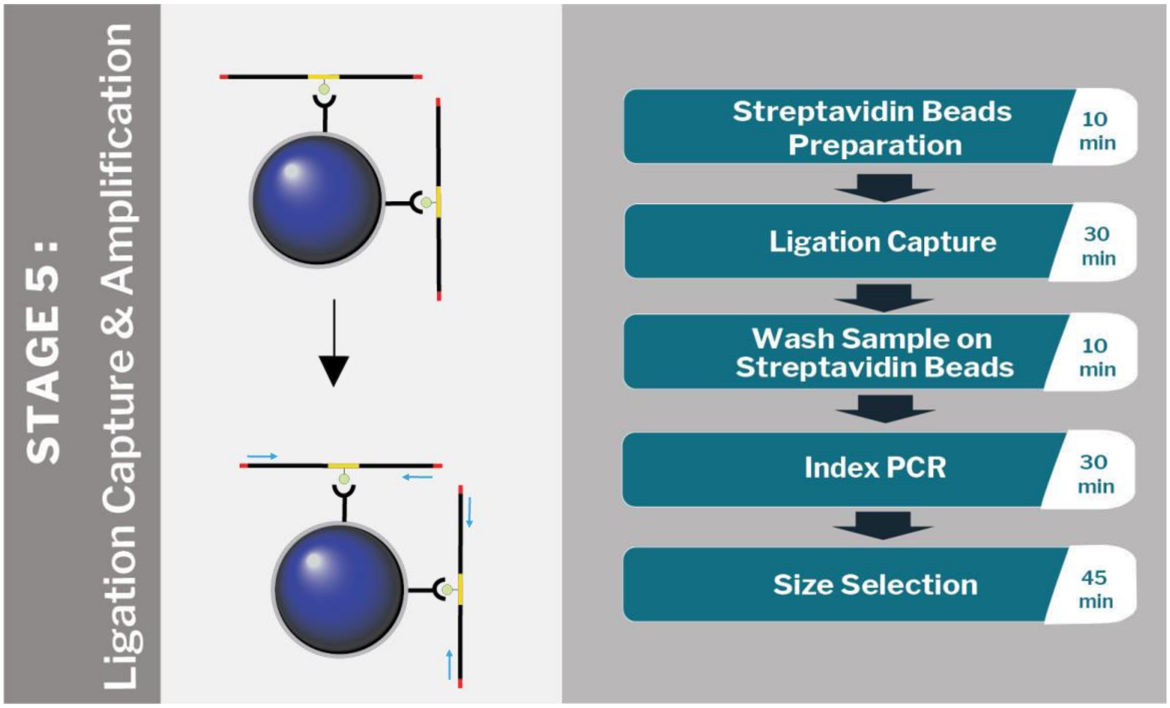
Stage 5: Ligation Capture and Amplification

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

☐ The Ligation Capture and Amplification protocol takes ~ 2 hours.

☐ Follow best practices when working with beads (see Good Practices).

Figure 6. Stage 5: Ligation Capture and Amplification



Before You Begin

☐ Thaw the index primers and HotStart PCR Ready Mix and keep **on** ice while in use. Pipet to mix prior to use.

☐ Equilibrate Streptavidin Beads and NWB at room temperature.

5.1 Streptavidin Beads Preparation

☐ NOTE This step does not involve any DNA sample.

Follow the steps below for Streptavidin Beads Preparation:

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 1 minute (or until the solution looks clear). Discard the supernatant.
3. Remove the tube from the magnetic rack and wash the beads with 200 µL of 1X Wash Buffer: pipet up and down to resuspend the beads and place the tube in the magnetic rack for 1 minute (or until the solution looks clear). 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 **NWB.** Pipet up and down to fully mix.

5.2 Ligation Capture

Follow the steps below for Ligation Capture:

1. Transfer the 100 µL of prepared Streptavidin beads (from Step 5, Stage 5.1) to the 0.2 mL PCR tube containing the 20 µL of purified adaptor-ligated DNA (from step 13 in 4.3 DNA Purification).
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 (or on the bench with no shaking, if an agitating thermal mixer for 0.2 mL tubes is not available).

5.3 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 (or until the solution looks clear), 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 **twice** with 200 µL NWB.
3. Wash the beads **twice** with 200 µL 1X Wash Buffer.

5.4 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 40 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	15 µL	16.5 µL	x	8	=	132 µL
HotStart PCR Ready Mix	25 µL	27.5 µL	x	8	=	220 µL
Total	40 µL					

2. Add 5 µL of DG i5 Index Primer to the PCR reaction. (see Appendix 1: Index Primers).
3. Add 5 µL of DG i7 Index Primer to the PCR reaction. (see Appendix 1: Index Primers).
4. Pipet up and down to fully mix.
5. 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	12
Anneal	65°C	30 seconds	
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	

5.5 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. 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 (or until the solution looks clear).
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.

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

Sequencing & QC Analysis of Dovetail® Micro-C Libraries

Dovetail® Micro-C libraries are sequenced via Illumina® sequencers in paired-end mode. Each Micro-C library can be deep sequenced up to 300 M read pairs (2 x 150 bp) on any Illumina platform. We recommend shallow sequencing the library to run a QC analysis prior to deep sequencing. The QC analysis requires 1 to 2 million read pairs (2 x 150 bp) generated from **MiniSeq** or **MiSeq**. If you don't have access to a MiSeq or MiniSeq, you can shallow sequence the library on an alternative Illumina platform to assess mappability and long-range information. However, you may not be able to accurately assess the duplication rate. Cantata Bio provides all kit users with access to QC analysis pipeline available on readthedocs (https://micro-c.readthedocs.io/en/latest/index.html).

Appendix 1: Index Primers

Dovetail® Primer Module Set 1 includes four i5 index primers and six i7 index primers. **Verify that the indexes selected for pooling have the appropriate color balance for your sequencing instrument.**

Dovetail® Primer Module Set 1 contains sufficient index primers to support multiplexing up to 24 libraries, where each library is prepared by combining a unique i5 index primer and a unique i7 index primer in steps 2 and 3 of Stage 5.4 Index PCR. For example, 6 libraries can be prepared by setting up 6 individual PCR reactions as follows:

- Library 1: i5 Index Primer 1 and i7 Index Primer 1
- Library 2: i5 Index Primer 1 and i7 Index Primer 2
- Library 3: i5 Index Primer 1 and i7 Index Primer 3
- Library 4: i5 Index Primer 1 and i7 Index Primer 4
- Library 5: i5 Index Primer 1 and i7 Index Primer 5
- Library 6: i5 Index Primer 1 and i7 Index Primer 6

Table 7. Index Primer

i5 Index Name	Bases in Adapter	Bases for Sample Sheet in Forward Orientation	Bases for Sample Sheet in Reverse Complement Orientation
DG i5 Index 1	ATATGCGC	ATATGCGC	GCGCATAT
DG i5 Index 2	TGGTACAG	TGGTACAG	CTGTACCA
DG i5 Index 3	AACCGTTC	AACCGTTC	GAACGGTT
DG i5 Index 4	TAACCGGT	TAACCGGT	ACCGGTTA

i7 Index Name	Bases in Adapter	Bases for Sample Sheet
DG i7 Index 1	ACGATCAG	CTGATCGT
DG i7 Index 2	TCGAGAGT	ACTCTCGA
DG i7 Index 3	CTAGCTCA	TGAGCTAG
DG i7 Index 4	ATCGTCTC	GAGACGAT
DG i7 Index 5	TCGACAAG	CTTGTCGA
DG i7 Index 6	CCTTGGAA	TTCCAAGG

Appendix 2: Troubleshooting Guide

Refer to this troubleshooting guide if the percentage of mononucleosomes of your sample is < 20% (i.e. chromatin is under-digested). The steps below allow you to re-digest the sample when additional sample is not available.

1. Thaw the digested sample at room temperature, if stored at -80°C (step 1 NOTE in Stage 2: Sample Preparation QC).
2. Pipet mix the digested sample then spin at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
3. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
4. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant.
5. Resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin).
6. Add 2 µL of MNase Enzyme Mix. Pipet up and down to fully mix.
7. 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.
8. Stop the reaction by adding 5 µL of 0.5 M EGTA. Pipet up and down to fully mix.
9. Continue to Stage 2: Sample Preparation QC.

Appendix 3: Cryopreservation

A. Cryopreserve Peripheral Blood Mononuclear Cells (PBMCs) using CryoStor®

As you prepare for cryopreservation, keep the following in mind:

- At any point in the protocol if there are cell aggregations observed, it is essential to filter out the aggregates using MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

Before You Begin

☐ Ensure CryoStor® is stored on ice.

Follow the steps below for Cryopreservation:

1. Wipe down the outside of the CryoStor® CS10 container with 70% ethanol before opening the bottle.
2. Centrifuge cells at 500 x g for 5 minutes.
3. Carefully pipette out the supernatant, leaving a small amount of liquid to ensure the cell pellet is not disturbed.
4. Resuspend the cell pellet by gently flicking the tube.
5. Add cold (2 - 8°C) CryoStor® CS10, pipet up and down to mix thoroughly.
6. Transfer the suspension to a cryovial.

> NOTE It is recommended to freeze isolated PBMCs at a concentration of 2 – 10 x 10^6 cells/mL.

7. Incubate the tube at 2 - 8°C for 10 minutes.
8. If using Nalgene® Mr. Frosty, transfer the tube in the Mr. Frosty and place in a -80°C freezer overnight.

> NOTE An alternative method could be used for rate-controlled cooling to approximately -1°C /minute in a controlled-rate freezer.

9. For long-term storage, transfer vials of frozen PBMCs from the freezer to vapor phase liquid nitrogen (below -135°C).

B. Cryopreserve Peripheral Blood Mononuclear Cells (PBMCs) using 90% FBS/10% DMSO

As you prepare for cryopreservation, keep the following in mind:

- At any point in the protocol if there are cell aggregations observed, it is essential to filter out the aggregates using MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be in single-cell suspension in the tube.

Before You Begin

☐ Ensure DMSO is at room temperature.
☐ Fetal Bovine Serum (FBS) should be stored on ice.

Follow the steps below for Cryopreservation:

1. Prepare 1 mL of 10% DMSO in FBS in a 1.5 mL tube. Keep on ice.
2. Ensure PBMCs are in single-cell suspension.
3. Spin the cells at 500 x g for 5 minutes at room temperature.
4. Discard the supernatant, leaving a small amount of buffer to ensure the cell pellet is not disturbed.
5. Resuspend the pellet in 10% DMSO-FBS mix. Pipet up and down to break up clumps and resuspend the pellet.

NOTE It is recommended to freeze isolated PBMCs at a concentration of 2– 10 x 10^6 cells/mL.

6. Transfer the suspension to a cryovial.
7. If using Nalgene® Mr. Frosty, transfer the tube in the Mr. Frosty and place in a -80°C freezer overnight.

NOTE An alternative method could be used for rate-controlled cooling to approximately -1°C /minute in a controlled-rate freezer.

8. For long-term storage, transfer vials of frozen PBMCs from the freezer to vapor phase liquid nitrogen (below -135°C).