



# Dovetail® LinkPrep™ Kit USER GUIDE

Version 1.0  
November 4, 2024

## Copyright

Use of this product is subject to compliance with applicable Cantata Bio, LLC terms and licensing requirements described at <https://dovetailgenomics.com/terms-and-conditions/>.

Dovetail®, Dovetail Genomics®, and HiRise™ are trademarks of Dovetail Genomics, LLC, a wholly owned subsidiary of Cantata Bio, LLC, in the U.S. and/or other countries. Illumina®, HiSeq®, MiSeq®, MiniSeq®, iSeq®, NextSeq®, NovaSeq® are registered trademarks of Illumina, Inc. Beckman Coulter™ and SPRIselect® are trademarks or registered trademarks of Beckman Coulter, Inc. SepMate™ is a registered trademark of StemCell Technologies Inc. Lymphoprep™ is a trademark of Serumwerk Bernburg AG. CryoStor® is a registered trademark of BioLife Solutions, Inc. Qubit® and eBioscience™ 10X RBC Lysis Buffer are trademarks or registered trademarks of Thermo Fisher Scientific. TapeStation® and Bioanalyzer® Systems are registered trademarks of Agilent Technologies.

This documentation shall be used only by Cantata Bio, LLC customers in connection with the use of the Dovetail® LinkPrep™ Kit and shall not be used for any other purpose without the prior written consent of Cantata Bio, LLC.

This document, the associated kits, and their contents shall not be used, distributed, or reproduced in whole or in part and/or otherwise communicated or disclosed without the prior written consent of Cantata Bio, LLC.

Prior to using Dovetail® LinkPrep™ Kit, these instructions must be read and understood by properly trained personnel. FAILURE TO FOLLOW THESE INSTRUCTIONS MAY RESULT IN FAILURE OF THE PRODUCT TO PERFORM AS EXPECTED, DAMAGE TO THE PRODUCT OR CUSTOMER SAMPLES, INJURY TO PERSONS, INCLUDING TO USERS OR OTHERS, AND DAMAGE TO OTHER PROPERTY. CANTATA BIO, LLC, DOES NOT ASSUME ANY LIABILITY ARISING OUT OF THE IMPROPER USE OF THE DOVETAIL® LINKPREP™ KIT. The contents of this product have been specifically designed to work together and are optimized for this protocol. Please do not make any substitutions.

The use of this product may require the buyer to obtain additional third-party intellectual property rights for certain applications. Safety Data Sheets are available at <https://dovetailgenomics.com/support/sds/>.

For Research Use Only. Not for use in diagnostics or diagnostic procedures.  
© 2022 Cantata Bio, LLC. All rights reserved.

### **For technical customer support, please contact**

[support@cantatabio.com](mailto:support@cantatabio.com)

+1 (831) 233-3780

## Dovetail® LinkPrep™ Kit Components and Storage

**Note: The tube label indicates a volume sufficient for 8 reactions, however the volume supplied in the tube includes an overage to account for pipetting carryover.**

### LinkPrep™ Proximity Core 4°C

Components	Volume	Storage
10X Wash Buffer	15 mL	
Chromatin Capture Beads	1,200 µL	
Fragmentation Stop	8 µL	2°C to 8°C
Crosslink Reversal Buffer	800 µL	
Nuclei Prep Buffer	800 µL	

### LinkPrep™ Proximity Core -20° C

Components	Volume	Storage
Fragmentation Enzyme Mix	20 µL	
4X Fragmentation Buffer	100 µL	
Proximity Ligation 1 Enzyme Mix	20 µL	
5X Proximity Ligation 1 Buffer	80 µL	
Proximity Ligation 2 Enzyme Mix	40 µL	-30°C to -10°C
5X Proximity Ligation 2 Buffer	80 µL	
Proximity Ligation 3 Enzyme Mix	8 µL	
5X Proximity Ligation 3 Buffer	80 µL	
Proteinase K	24 µL	

### LinkPrep™ Library Module for Illumina

Components	Volume	Storage
Library Prep 1 Enzyme Mix	12 µL	
5X Library Prep 1 Buffer	80 µL	
Library Prep 2 Enzyme Mix	8 µL	
2X Library Prep 2 Buffer	400 µL	-30°C to -10°C
Library Prep 3 Enzyme Mix	20 µL	
HotStart PCR Ready Mix	200 µL	

**LinkPrep™ Primer Module Set #1**

Components	Volume	Storage
I5 Index Primer 1	30 µL	
I5 Index Primer 2	30 µL	
I5 Index Primer 3	30 µL	
I5 Index Primer 4	30 µL	
I7 Index Primer 1	20 µL	-30°C to -10°C
I7 Index Primer 2	20 µL	
I7 Index Primer 3	20 µL	
I7 Index Primer 4	20 µL	
I7 Index Primer 5	20 µL	
I7 Index Primer 6	20 µL	

**Dovetail® Cell Isolation Module**

Components	Volume/Mass	Storage
Cell Isolation Enzyme Mix (powder)	45 mg	
Reconstitution Buffer	1,700 µL	2°C to 8°C*
100mM CaCl <sub>2</sub>	200 µL	

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

## User Supplied Reagents, Consumables and Equipment

### Reagents

Reagent	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
DSG (Disuccinimidyl Glutarate)	Thermo Fisher Scientific	A35392
DMSO (Dimethyl Sulfoxide, Anhydrous ≥ 99.99%)	Sigma-Aldrich	276855-100ML
TE Buffer pH 8.0	Thermo Fisher Scientific	AM9849
10% Triton X-100	Thermo Fisher Scientific	85111
SepMate™-15*	Stemcell technologies	85415
Lymphoprep™*	Stemcell technologies	07801
eBioscience™ 10X RBC Lysis Buffer**	Thermo Fisher Scientific	00-4300
CryoStor®***	BioLife Solutions	210373

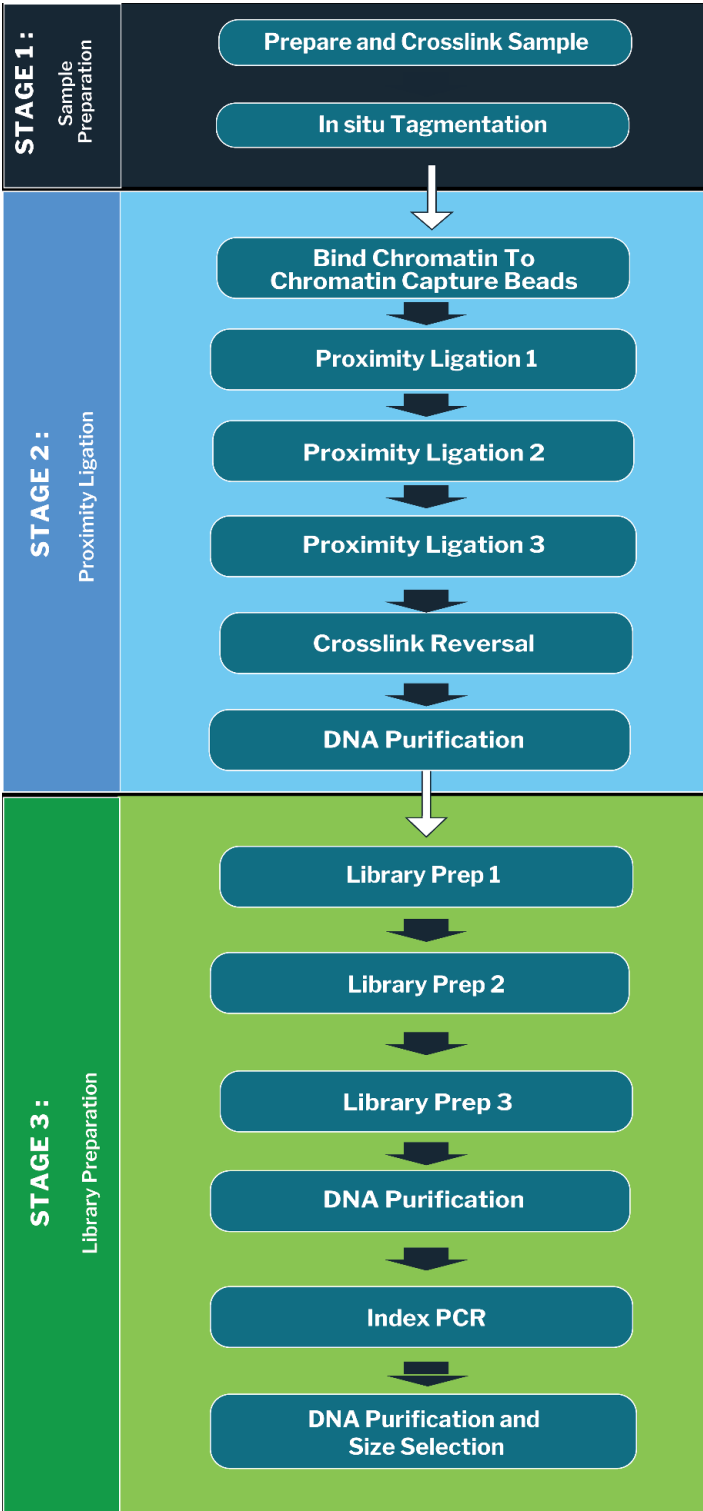
\*only needed if working with Fresh Mammalian Whole Blood (≤ 24 hours post collection)

\*\*only needed if working with Fresh Mammalian Whole Blood (24-72 hours post collection)

\*\*\*optional for cryopreserving PBMCs

Consumable/Equipment	Supplier	Part Number
1.5 mL Low binding microcentrifuge tubes		
0.2 mL PCR tubes		
Pipets and pipet tips		
Magnetic separation rack for 0.2 mL and 1.5 mL tubes		
Agitating thermal mixer	Generic	
Thermal cycler		
Vortex mixer		
Centrifuge for 0.2 mL and 1.5 mL tubes		
Hemocytometer		
Swinging bucket rotor		
MiniStrainer 70 µm mesh	pluriSelect	43-10070-46
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 of Bioanalyzer)	Agilent	Various

# Dovetail® LinkPrep™ Protocol Overview



## Good Practices

- Read the entire guide before use, including Before You Begin and the Notes.
- The cell input amount will influence the efficiency of the fragmentation 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 crosslinking. **Ensure the centrifuge is set to 500 x g or 500 x rcf (NOT rpm).**
- To ensure efficient crosslinking, a new or recently opened solution of formaldehyde should be used. Formaldehyde solution containing white precipitates should not be used.
- Keep all enzymes and master mixes on ice during setup and use. Promptly move reagents back to the indicated storage.
- Fully thaw buffers, place on ice and thoroughly mix before use.
- Always add the reagents to the master mix in the specified order as listed throughout the protocol.
- When preparing master mixes, scale the volume of each reagent as appropriate, using 10% excess volume to compensate for pipetting loss.
- When working with beads, such as Chromatin Capture, 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.
- Cantata Bio's list of validated samples is not limited to and includes:

Validated cell lines	Validated mammalian tissues
GM12878	Blood / PBMCs
HG002	Liver
K562	Brain
Detroit562	Lung
HT-1197	Spleen
LNCaP	Heart
HCC1187	Quadricep muscle
HEK-293T	Colon
Colo829	Kidney
NIH-3T3	Prostate
N2A	Testes

## Stage 1A: Sample Preparation - Mammalian Cell Lines

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

- Sample preparation takes ~ 1 hour and 30 minutes.

### Before You Begin

- The 10X Wash Buffer and Fragmentation Stop 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 the 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.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use.** Make sure there is no precipitate visible. Vortex to mix prior to use.
- **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.
- If working with multiple samples, you may choose **step 11 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

Follow the steps below for Sample Preparation:

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot  $1 \times 10^6$  cells into a 1.5 mL tube.
3. Spin the  $1 \times 10^6$  cell aliquot at  $500 \times g$  for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet. To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.

### NOTES

- The cell centrifugation steps (5 – 11) must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.
- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- All crosslinking reactions (steps 5 – 11) should be carried out at room temperature.
- You may choose to freeze your cell pellet at  $-80^{\circ}\text{C}$  at this stage. Otherwise, proceed with the protocol.



4. Resuspend the cell pellet in 50  $\mu\text{L}$  of 1X PBS and pipet up and down to ensure no clumps are present then add 150  $\mu\text{L}$  of 1X PBS to bring the volume to 200  $\mu\text{L}$ . Pipet up and down to fully resuspend the pellet.
5. Add 2  $\mu\text{L}$  of 0.3 M DSG, pipet to mix.
6. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
7. Add 5.4  $\mu\text{L}$  of 37% formaldehyde.
8. Rotate the tube for 10 minutes at room temperature. Cells 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, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

10. Wash the pellet with 150  $\mu\text{L}$  of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
11. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

#### NOTES

- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- This is a SAFE HOLD step: after discarding the supernatant, you can leave your tube on ice if you need to continue processing other samples before moving to step 12 with all samples in parallel.

12. Resuspend the pellet in 52.5  $\mu\text{L}$  of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
Reconstituted Cell Isolation Enzyme Mix (see Before You Begin)	50 $\mu\text{L}$
100mM $\text{CaCl}_2$	2.5 $\mu\text{L}$
<b>Total</b>	<b>52.5 <math>\mu\text{L}</math></b>

13. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
37°C	10 minutes

14. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

15. Resuspend the cells in 100  $\mu\text{L}$  Nuclei Prep Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

16. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
62°C	10 minutes

17. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE** To minimize cell loss, remove the supernatant **SLOWLY** from the **TOP DOWN**, using a **P200 pipette**.

18. Resuspend the cell pellet in 52.5 µL of the fragmentation master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	37.5 µL
4X Fragmentation Buffer	12.5 µL
Fragmentation Enzyme Mix	2.5 µL
<b>Total</b>	<b>52.5 µL</b>

19. Pipet up and down to fully mix. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
55°C	20 minutes

**NOTE** Precipitation may occur during this reaction, this is normal.

20. Quick spin the tube. Stop the reaction by adding 1 µL of **Fragmentation Stop**. **Pipet up and down to fully mix**.

21. Incubate the tube in the thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
65°C	5 minutes

22. Quick spin the tube. Add 6 µL of 10% Triton. **Pipet up and down to fully mix**.

23. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE**.

24. Aliquot 2.5 µL of the sample into a new tube labeled QC and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

**NOTE** The QC tube containing the 2.5 µL aliquot can be stored at -80°C for 6 months and used in troubleshooting, if needed.

## Stage 1B: Sample Preparation - Fresh frozen mammalian tissues

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

- Tissue sample preparation takes ~ 2 hours.

### Before You Begin

- The 10X Wash Buffer and Fragmentation Stop 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 the 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.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use.** Make sure there is no precipitate visible. Vortex to mix prior to use.
- **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.
- **Check out this [video](#) which showcases proper grinding and correct consistency of the ground tissue in step 2.**
- If working with multiple samples, you may choose **step 11 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

Follow the steps below for Sample Preparation:

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

Tissue	Input Mass	Cell Isolation Module? (steps 12-14)
Brain	5 mg	No
Liver	5 mg	No
Testes	5 mg	No
Lung	5 mg	Yes
Kidney	5 mg	Yes
Colon	5 mg	Yes
Spleen	3 mg	Yes
Heart	5 mg	Yes
Muscle	20 mg	Yes

2. Disrupt the tissue by grinding it to a **fine powder** with a mortar and pestle **in liquid nitrogen**.
3. Transfer the ground tissue to a 1.5 mL tube.

### NOTES

- You may choose to freeze your ground/disrupted 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  $\mu\text{L}$  of 1X PBS and pipet up and down to ensure no clumps are present.
5. Add 2  $\mu\text{L}$  of 0.3 M DSG. Pipet to mix.
6. Rotate the tube for 10 minutes at room temperature. Sample should not settle.
7. Add 5.4  $\mu\text{L}$  of 37% formaldehyde.
8. Rotate the tube for 10 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, taking care not to disturb the pellet. To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.

#### NOTES

- The centrifugation steps must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.
- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

10. Wash the tissue pellet with 100  $\mu\text{L}$  of 1 X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
11. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

#### NOTES

- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- This is a SAFE HOLD step: after discarding the supernatant, you can leave your tube on ice if you need to continue processing other samples before moving to step 12 with all samples in parallel.

**For tissues requiring Cell Isolation Module** (see table above) perform steps 12-14, otherwise proceed to step 15.

12. Resuspend the tissue pellet in 105  $\mu\text{L}$  of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
Reconstituted Cell Isolation Enzyme Mix (see Before You Begin)	100 $\mu\text{L}$
100 mM $\text{CaCl}_2$	5 $\mu\text{L}$
<b>Total</b>	<b>105 <math>\mu\text{L}</math></b>

13. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
37°C	<b>30 minutes</b>

- Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

- Resuspend the pellet in 100 µL Nuclei Prep Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
- Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
62°C	10 minutes

- Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

- Resuspend the pellet in 52.5 µL of the fragmentation master mix containing the following reagents **added in the order listed:**

Reagent	Volume Per Reaction
UltraPure Water	37.5 µL
4X Fragmentation Buffer	12.5 µL
Fragmentation Enzyme Mix	2.5 µL
<b>Total</b>	<b>52.5 µL</b>

- Pipet up and down to fully mix. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
55°C	20 minutes

**NOTE** Precipitation may occur during this reaction, this is normal.

- Quick spin the tube. Stop the reaction by adding 1 µL of **Fragmentation Stop. Pipet up and down to fully mix.**
- Incubate the tube in the thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
65°C	5 minutes

- Quick spin the tube. Add 6 µL of 10% Triton. **Pipet up and down to fully mix.**
- Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE**.
- For muscular tissues** (such as quadricep, gastrocnemius, tibialis, heart, or tail) filter your sample through the 70 µm MiniStrainer to remove 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 using 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 25.**
- Aliquot 2.5 µL of the sample into a new tube labeled QC tube and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

**NOTE** The QC tube containing the 2.5 µL aliquot can be stored at -80°C for 6 months and used in troubleshooting, if needed.

## Stage 1C: Sample Preparation - Cryo-preserved Peripheral Blood Mononuclear Cells (PBMCs)

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

- Sample preparation takes ~ 1 hour and 30 minutes.

### Before You Begin

- The 10X Wash Buffer and Fragmentation Stop 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 the 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 ~15 mL of 1X Wash Buffer per sample for the entire protocol.
  - >> Pre-warm 1X Wash Buffer to 37°C for 15 minutes.
- **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.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use.** Make sure there is no precipitate visible. Vortex to mix prior to use.
- **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.
- If working with multiple samples, you may choose **step 15 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

Follow the steps below for Sample Preparation:

1. Quickly thaw cryopreserved PBMCs stock in a 37°C water bath for approximately 2-5 minutes.
2. Prepare a 15 mL tube containing 10 mL of **pre-warmed** 1X Wash Buffer.
3. Transfer the thawed PBMC mix to the tube containing the prewarmed 1X Wash buffer. Take care to pipet gently.
4. Spin the cells at 500 x g for 5 minutes. Discard the supernatant.
5. Resuspend the pellet in 200 µL of 1X PBS, pipet up and down 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.

6. 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.**
7. Pipette mix the PBMCs on ice. Transfer a volume equivalent to  $1 \times 10^6$  cells to a new 1.5 mL tube.

## NOTES

- The cell centrifugation steps must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.
- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- All crosslinking reactions (steps 9 – 15) should be carried out at room temperature.
- You may choose to cryopreserve remaining PBMCs in CryoStor® or DMSO and FBS at -80°C at this stage (Appendix 6). Otherwise, proceed with the protocol.

8. Bring up the volume to 200 µL with 1X PBS.
9. Add 2 µL of 0.3 M DSG, pipet to mix.
10. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
11. Add 5.4 µL of 37% formaldehyde.
12. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
13. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

14. Wash the pellet with 150 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
15. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

## NOTES

- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- This is a SAFE HOLD step: after discarding the supernatant, you can leave your tube on ice if you need to continue processing other samples before moving to step 16 with all samples in parallel.

16. Resuspend the pellet in 52.5 µL of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
Reconstituted Cell Isolation Enzyme Mix (see Before You Begin)	50 µL
100mM CaCl <sub>2</sub>	2.5 µL
<b>Total</b>	<b>52.5µL</b>

17. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
37°C	10 minutes

18. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

19. Resuspend the cells in 100 µL Nuclei Prep Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

20. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
62°C	10 minutes

21. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

22. Resuspend the cell pellet in 52.5 µL of the fragmentation master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	37.5 µL
4X Fragmentation Buffer	12.5 µL
Fragmentation Enzyme Mix	2.5 µL
<b>Total</b>	<b>52.5 µL</b>

23. Pipet up and down to fully mix. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
55°C	20 minutes

24. Quick spin the tube. Stop the reaction by adding 1 µL of **Fragmentation Stop**. **Pipet up and down to fully mix.**

**NOTE** Precipitation may occur during this reaction, this is normal.

25. Incubate the tube in the thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
65°C	5 minutes

26. Quick spin the tube. Add 6 µL of 10% Triton. **Pipet up and down to fully mix.**

27. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE**.

28. Aliquot 2.5 µL of the sample into a new tube labeled QC and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

**NOTE** The QC tube containing the 2.5 µL aliquot can be stored at -80°C for 6 months and used in troubleshooting, if needed.



## Stage 1D: Sample Preparation - Fresh Mammalian Whole blood ≤ 24h post collection

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

- Blood sample preparation takes ~ 2 hours.
- This protocol is validated for fresh blood collected in **EDTA, ACDA or Heparin tubes**.

### Before You Begin

- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood ≤ 24 hours post collection using SepMate™ isolation tubes.
  - > SepMate™-15 is designed to process 0.5 to 5 mL of blood samples. Please follow manufacturer's guidelines for volume recommendation.
  - > Warm Lymphoprep™ to room temperature (15 - 25°C) before use.
  - > Typically, 0.5 to 3 x 10<sup>6</sup> PBMCs are isolated from 1 mL of healthy whole blood.
  - > Fresh blood should be **stored and shipped at ambient temperature**. Blood samples processed within 24 hours of collection yield higher quality and quantity of PBMCs.
  - > It is essential to work quickly and limit handling of the PBMCs once they are isolated from whole blood.
- The 10X Wash Buffer and Fragmentation Stop 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 the 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.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use**. Make sure there is no precipitate visible. Vortex to mix prior to use.
- **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.
- If working with multiple samples, you may choose **step 23 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

Follow the steps below for Sample Preparation:

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.

- Pour the top layer containing the PBMCs into a new 1.5 mL tube.

**NOTE** Do not hold the tube in the inverted position for longer than 2 seconds.

- Add equal volume of 1X PBS to dilute the enriched PBMCs. Invert to mix.
- Centrifuge the PBMC containing tube at 500 x g for 5 minutes. Discard the supernatant.
- Resuspend the pellet in 200  $\mu$ L of 1X Wash Buffer, 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.

- Centrifuge at 500 x g for 5 minutes. Carefully discard the supernatant.
- Resuspend the pellet with 200  $\mu$ L of 1X PBS, pipet up and down gently to break up clumps and resuspend the pellet.
- Take an aliquot to count the cells. Keep the remaining cells on ice until cells are counted. **Count quickly, this is a delicate sample.**
- Gently, pipette mix the PBMCs on ice. Transfer  $1 \times 10^6$  cells into a new 1.5 mL tube.

#### NOTES

- The cell centrifugation steps must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.
- To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- All crosslinking reactions (steps 17 – 23) should be carried out at room temperature.
- You may choose to cryopreserve remaining PBMCs in CryoStor<sup>®</sup> or DMSO and FBS at -80°C at this stage (Appendix 6). Otherwise, proceed with the protocol.

- Bring up the volume to 200  $\mu$ L of 1X PBS, if needed.
- Add 2  $\mu$ L of 0.3 M DSG. Pipet to mix.
- Rotate the tube for 10 minutes at room temperature. Cells should not settle.
- Add 5.4  $\mu$ L of 37% formaldehyde.
- Rotate the tube for 10 minutes at room temperature. Cells should not settle.
- Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet. To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.
- Wash the pellet with 150  $\mu$ L 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, taking care not to disturb the pellet.

#### NOTES

- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- This is a SAFE HOLD step: after discarding the supernatant, you can leave your tube on ice if you need to continue processing other samples before moving to step 24 with all samples in parallel.

24. Resuspend the pellet in 52.5  $\mu$ L of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
Reconstituted Cell Isolation Enzyme Mix (see Before You Begin)	50 $\mu$ L
100mM CaCl <sub>2</sub>	2.5 $\mu$ L
<b>Total</b>	<b>52.5 <math>\mu</math>L</b>

25. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
37°C	10 minutes

26. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

27. Resuspend the cells in 100  $\mu$ L Nuclei Prep Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

28. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
62°C	10 minutes

29. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

30. Resuspend the pellet in 52.5  $\mu$ L of the fragmentation master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	37.5 $\mu$ L
4X Fragmentation Buffer	12.5 $\mu$ L
Fragmentation Enzyme Mix	2.5 $\mu$ L
<b>Total</b>	<b>52.5 <math>\mu</math>L</b>

31. Pipet up and down to fully mix. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
55°C	20 minutes

NOTE Precipitation may occur during this reaction, this is normal.

32. Quick spin the tube. Stop the reaction by adding 1  $\mu$ L of **Fragmentation Stop. Pipet up and down to fully mix.**

33. Incubate the tube in the thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
65°C	5 minutes

34. Quick spin the tube. Add 6 $\mu$ L of 10% Triton. **Pipet up and down to fully mix.**

35. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE.**

36. Aliquot 2.5  $\mu$ L of the sample into a new tube labeled QC and **store at -80°C.** Proceed with the rest of your sample to Proximity Ligation ( Stage 2).

NOTE The QC tube containing the 2.5  $\mu$ L aliquot can be stored at -80°C for 6 months and used in troubleshooting, if needed.

## Stage 1E: Sample Preparation - Fresh Mammalian Whole blood 24-72h post collection

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

- Blood sample preparation takes ~ 2 hours.
- This protocol is validated for fresh blood collected in **EDTA, ACDA or Heparin tubes**.

### Before You Begin

- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood 24-72 hours post collection using 10X RBC Lysis Buffer.
  - >> The eBioscience™ 10X RBC Lysis Buffer contains ammonium chloride which is optimal for lysis of erythrocytes with minimal effect on lymphocytes. This preparation method tends to contain higher amounts of cellular debris and may affect cell counting accuracy.
  - >> Warm eBioscience™ 10X RBC Lysis Buffer to room temperature (15 - 25°C) before use.
  - >> Typically, 0.5 to 3 x 10<sup>6</sup> PBMCs are isolated from 1 mL of healthy whole blood.
  - >> Fresh blood should be **stored and shipped at ambient temperature**. Blood samples processed within 24 hours of collection yield higher quality and quantity of PBMCs. Please see Stage 1C.
  - >> It is essential to work quickly and limit handling of the PBMCs once they are isolated from whole blood.
- The 10X Wash Buffer and Fragmentation Stop 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 the 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.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use**. Make sure there is no precipitate visible. Vortex to mix prior to use.
- **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.
- If working with multiple samples, you may choose **step 17 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

### Follow the steps below for Sample Preparation:

1. In a 50 mL tube, prepare 30 mL of 1X RBC Lysis Buffer by adding 3 mL of eBioscience™ 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.

**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.

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.
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.**
9. Gently, pipette mix the PBMCs on ice. Transfer  $1 \times 10^6$  cells into a new 1.5 mL tube.

#### NOTES

- The cell centrifugation steps must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.
- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- All crosslinking reactions (steps 11 – 15) should be carried out at room temperature.
- You may choose to cryopreserve remaining PBMCs in CryoStor® or DMSO and FBS at -80°C at this stage (Appendix 6). Otherwise, proceed with the protocol.

10. Bring up the volume to 200 µL of 1X PBS, if needed.
11. Add 2 µL of 0.3 M DSG. Pipet to mix.
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 for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet. To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

16. Wash the pellet with 150 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, taking care not to disturb the pellet.

#### NOTES

- **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**
- This is a SAFE HOLD step: after discarding the supernatant, you can leave your tube on ice if you need to continue processing other samples before moving to step 18 with all samples in parallel.

18. Resuspend the pellet in 52.5 µL of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
Reconstituted Cell Isolation Enzyme Mix (see Before You Begin)	50 $\mu$ L
100mM CaCl <sub>2</sub>	2.5 $\mu$ L
<b>Total</b>	<b>52.5 <math>\mu</math>L</b>

19. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
37°C	10 minutes

20. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

21. Resuspend the cells in 100  $\mu$ L Nuclei Prep Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
22. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
62°C	10 minutes

23. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant, taking care not to disturb the pellet.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

24. Resuspend the pellet in 52.5  $\mu$ L of the fragmentation master mix containing the following reagents **added in the order listed:**

Reagent	Volume Per Reaction
UltraPure Water	37.5 $\mu$ L
4X Fragmentation Buffer	12.5 $\mu$ L
Fragmentation Enzyme Mix	2.5 $\mu$ L
<b>Total</b>	<b>52.5 <math>\mu</math>L</b>

25. Pipet up and down to fully mix. Incubate the tube in a thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
55°C	20 minutes

**NOTE** Precipitation may occur during this reaction, this is normal.

26. Quick spin the tube. Stop the reaction by adding 1  $\mu$ L of **Fragmentation Stop. Pipet up and down to fully mix.**
27. Incubate the tube in the thermal mixer WITHOUT SHAKING as follows:

Temperature	Time
65°C	5 minutes

28. Quick spin the tube. Add 6 $\mu$ L of 10% Triton. **Pipet up and down to fully mix.**
29. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE.**
30. Aliquot 2.5  $\mu$ L of the sample into a new tube labeled QC tube and **store at -80°C.** Proceed with the rest of your sample to Proximity Ligation ( Stage 2).

**N O T E** The QC tube containing the 2.5  $\mu$ L aliquot can be stored at -80°C for 6 months and used in troubleshooting, if needed.



## Stage 2: Proximity Ligation

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

- Proximity ligation takes ~ 1.5 hour.
- 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 prior to use.
- Follow best practices when working with beads (see Good Practices). The below protocol uses 0.2 mL strip tubes and corresponding magnetic rack. However, the below protocol is compatible with 1.5 mL tubes and corresponding magnetic rack.

### Before You Begin

- Thaw 5X Proximity Ligation 1 Buffer, 5X Proximity Ligation 2 Buffer, 5X Proximity Ligation 3 Buffer at room temperature. Leave on ice once thawed. Vortex to mix prior to use.
- Equilibrate Chromatin Capture Beads to room temperature.

## 2.1 Bind Chromatin to Chromatin Capture Beads

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

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Transfer 100  $\mu\text{L}$  of resuspended Chromatin Capture Beads to a **new** 0.2 mL PCR tube.
3. Add your lysate to the Chromatin Capture Beads aliquoted above.
4. Pipet up and down to fully mix. Incubate at room temperature, off the magnetic rack, for 10 minutes.
5. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Discard the supernatant.
6. Remove the tube from the magnetic rack and wash the beads with 150  $\mu\text{L}$  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.
7. Remove the tube from the magnetic rack and resuspend the beads in 150  $\mu\text{L}$  1X Wash Buffer. Pipet up and down to fully resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

## 2.2 Proximity Ligation 1

Follow the steps below for Proximity Ligation 1:

1. Prepare the proximity ligation 1 master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	40 $\mu\text{L}$
5X Proximity Ligation 1 Buffer	10 $\mu\text{L}$
Proximity Ligation 1 Enzyme Mix	2.5 $\mu\text{L}$
<b>Total</b>	<b>52.5 <math>\mu\text{L}</math></b>

2. Place the tube from [step 7 in Stage 2.1](#) 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 52.5  $\mu\text{L}$  of the proximity ligation 1 master mix.

- Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,500 rpm as follows:

Temperature	Time
25°C	15 minutes

- Quick spin the tube and place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
- Remove the tube from the magnetic rack and resuspend the beads in 150  $\mu$ L 1X Wash Buffer. Pipet up and down to fully resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

## 2.3 Proximity Ligation 2

Follow the steps below for Proximity Ligation 2:

- Prepare the proximity ligation 2 master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	40 $\mu$ L
5X Proximity Ligation 2 Buffer	10 $\mu$ L
Proximity Ligation 2 Enzyme Mix	5 $\mu$ L
<b>Total</b>	<b>55 <math>\mu</math>L</b>

- Place the tube from [step 6 in Stage 2.2](#) 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**).
- Remove the tube from the magnetic rack and **immediately** add to the beads 55  $\mu$ L of the proximity ligation 2 master mix.
- Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,500 rpm as follows:

Temperature	Time
37°C	15 minutes

- Quick spin the tube and place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
- Remove the tube from the magnetic rack and resuspend the beads in 150  $\mu$ L 1X Wash Buffer. Pipet up and down to fully resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

## 2.4 Proximity Ligation 3

Follow the steps below for Proximity Ligation 3:

- Prepare the proximity ligation 3 master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
UltraPure Water	40 $\mu$ L
5X Proximity Ligation 3 Buffer	10 $\mu$ L
Proximity Ligation 3 Enzyme Mix	1 $\mu$ L
<b>Total</b>	<b>51 <math>\mu</math>L</b>

- Place the tube from [step 6 in Stage 2.3](#) 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**).
- Remove the tube from the magnetic rack and **immediately** add to the beads 51  $\mu$ L of the proximity ligation 3 master mix.

- Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,500 rpm as follows:

Temperature	Time
37°C	15 minutes

- Quick spin the tube and place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.

## 2.5 Crosslink Reversal

Follow the steps below for Crosslink Reversal:

- Remove the tube from the magnetic rack and add to the beads 51.5 µL of a master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
Crosslink Reversal Buffer	50 µL
Proteinase K	1.5 µL
<b>Total</b>	<b>51.5 µL</b>

- Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,500 rpm as follows:

Temperature	Time
78°C	10 minutes
25°C	Hold
Note: secure the tube lid to prevent opening during incubation.	

- Quick spin the tube and place it in the magnetic rack for 1 minute. **Transfer 50 µL of the SUPERNATANT** to a new 0.2 mL PCR tube. Discard the beads.

## 2.6 DNA Purification

Follow the steps below for DNA Purification:

- Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
- Add 90 µL of resuspended SPRIselect beads to the 0.2 mL PCR tube containing your sample.
- Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
- Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
- Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
- 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.
- 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.
- Air dry the beads for 3 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads.**
- Off the magnetic rack, resuspend the beads in 42 µL TE Buffer pH 8.0.
- Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
- Incubate at room temperature, off the magnetic rack, for 2 minutes.
- Quick spin the tube and place it in the magnetic rack for 1 minute.
- Transfer 40 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.

- We expect a yield  $\geq 150$  ng.
- If the yield you recover is  $\geq 150$  ng, proceed to Library Preparation (Stage 3).
- If the yield you recover is  $< 150$  ng, you should expect a lower complexity library which may be insufficient to support sequencing up to 300M read pairs. You have the following options:
  1. Continue with the Library Preparation (Stage 3), generate a library and shallow sequence it for QC analysis (see QC Analysis & Sequencing) to assess if the library you generated has sufficient complexity to support sequencing up to 300M read pair depth or if additional library is needed .
  2. Perform the Lysate QC and determine the lysate yield/your sample chromatin content (Refer to Appendix 2: Troubleshooting Guide For Yields).

SAFE STOP Purified DNA sample can be stored at  $-20^{\circ}\text{C}$  for up to 6 months.

## Stage 3: Library Preparation

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

- The library preparation protocol does not require sonication.
- The library preparation protocol takes ~ 3 hours.
- Follow best practices when working with beads (see Good Practices)

### Before You Begin

- Thaw 5X Library Prep 1 Buffer, 5X Library Prep 2 Buffer, HotStart PCR Ready Mix and Primers at room temperature. Leave on ice once thawed. Mix by inversion and quick spin prior to use.

### NOTES

- The protocol from here onwards is per **1 LIBRARY** prep. If you are preparing more than 1 library simultaneously, scale the reactions proportional to the number of libraries.
- LinkPrep Library Module **must be used** for library preparation. Other library preparation kits are **NOT** compatible.

### 3.1 Library Prep 1

Follow the steps below for Library Prep 1:

1. Place **up to 1,000 ng of purified DNA** from [step 13 Stage 2.6](#) in a new 0.2 mL PCR tube and bring the volume to 40  $\mu$ L with UltraPure Water.
2. Add to the tube the following reagents **in the order listed**:

Reagent	Volume Per Reaction
5X Library Prep 1 Buffer	10 $\mu$ L
Library Prep 1 Enzyme Mix	1.5 $\mu$ L
<b>Total</b>	<b>11.5 <math>\mu</math>L</b>

3. Pipet up and down to fully mix. Incubate in a thermal cycler as follows (lid at 75°C):

Temperature	Time
25°C	15 minutes
68°C	15 minutes

### 3.2 Library Prep 2

Follow the steps below for Library Prep 2:

1. Quick spin the tube. To the tube containing the reaction from library prep 1, add the following reagents **in the order listed**:

Reagent	Volume Per Reaction
2X Library Prep 2 Buffer	50 $\mu$ L
Library Prep 2 Enzyme Mix	1 $\mu$ L
<b>Total</b>	<b>51 <math>\mu</math>L</b>

2. Pipet up and down to fully mix. Incubate in a thermal cycler as follows (lid at 75°C):

Temperature	Time
25°C	15 minutes

### 3.3 Library Prep 3

Follow the steps below for Library Prep 3:

1. Quick spin the tube. To the tube containing the reaction from library prep 2, **add 2.5 µL of Lib Prep 3 Enzyme Mix to the tube.**
2. Pipet up and down to fully mix. Incubate in a thermal cycler as follows (lid at 75°C):

Temperature	Time
37°C	15 minutes

NOTE Thorough pipet mixing of the samples after addition of the Lib Prep 3 Enzyme Mix is very important. Pipet carefully as the solution is viscous.

### 3.4 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 0.2 mL PCR 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 5 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 3 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 16 µL UltraPure Water.
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 2 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. **Transfer** 15 µL of the **SUPERNTANT** (purified DNA) to a new 0.2ml tube. Discard the beads.

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

### 3.5 Index PCR

Follow the steps below for Index PCR:

1. Add 25 µL of the HotStart PCR Ready Mix to the tube from [step 13 in Stage 3.4](#).
2. Add 5 µL of I5 Index Primer to the PCR reaction (see Appendix 1: Dual Index Primers).
3. Add 5 µL of I7 Index Primer to the PCR reaction (see Appendix 1: Dual Index Primers).

- Pipet up and down to fully mix. Quick spin the tube, place the tube into the thermal cycler (lid temp: 105°C) and run the following program:

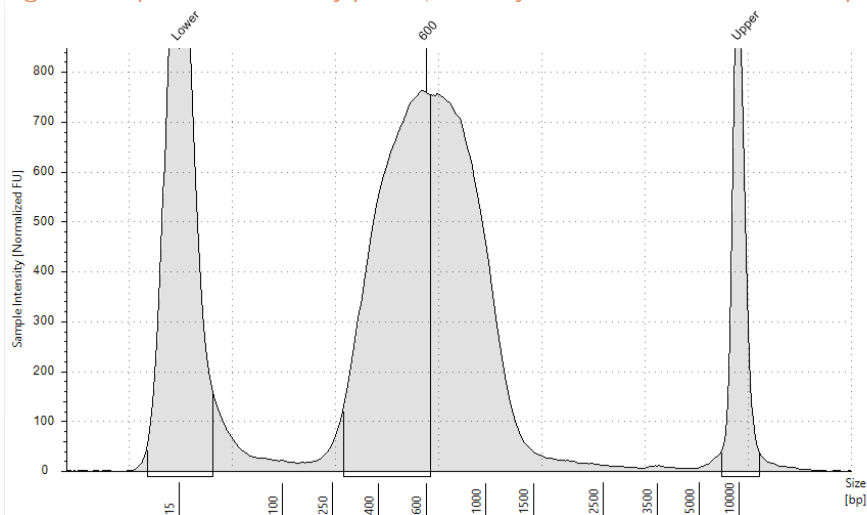
Step	Temperature	Time	Cycles
Enzyme Activation	98°C	3 minutes	1
Denature	98°C	20 seconds	
Anneal	60°C	30 seconds	12
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	

### 3.6 Size Selection

Follow the steps below for Size Selection:

- Quick spin the tube, add 50 µL of TE Buffer pH 8.0 to the tube to bring the volume of the sample in the tube to 100 µL.
- Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
- Add 50 µL of resuspended SPRIselect beads to the tube containing your sample.
- Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
- Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
- Quick spin the tube and place it in the magnetic rack for 5 minutes.
- Transfer 145 µL of the SUPERNATANT** to a new 0.2 mL PCR tube. Discard the beads.
- Add 30 µL of resuspended SPRIselect beads to the 0.2 mL PCR tube containing your sample.
- Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
- Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
- Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
- 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.
- 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.
- Air dry the beads for 3 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads.**
- Off the magnetic rack, resuspend the beads in 32 µL TE Buffer pH 8.0.
- Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
- Incubate the tube at room temperature, off the magnetic rack, for 2 minutes.
- Quick spin the tube and place it in the magnetic rack for 1 minute.
- Transfer** 30 µL of the **SUPERNATANT** to a new 0.2 mL PCR tube. The supernatant is your size selected library. Discard the beads.
- Quantify your size selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit. **We expect a final library yield > 200 ng if you used >150 ng input into library preparation. If the yield is < 200 ng, refer to the Troubleshooting Guide For Yields (Appendix 2).**
- 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 2).

Figure 2. Expected final library profile, as analyzed on HS D5000 ScreenTape



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

## QC Analysis & Sequencing

Dovetail® LinkPrep™ libraries are sequenced via Illumina® sequencers in paired-end mode. Each library can be deep sequenced to 300M read pairs (2 x 150 bp). The depth and number of libraries depend on the variants you want to detect and its tumor fraction and variant allele frequency (see table below). We recommend shallow sequencing the library to run a QC analysis prior to deep sequencing to determine the long-range information and complexity in the library. While this is recommended, the shallow sequencing for QC analysis is not required. The QC assessment is based on shallow sequenced data (1M - 10M read pairs 2 x 150 bp) generated by a **MiSeq** or **MiniSeq** platform. If you don't have access to a MiSeq or MiniSeq, you can shallow sequence the library on an alternative sequencing 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 access to QC analysis pipeline available on [readthedocs](https://readthedocs.org/projects/cantata-bio/).

	Tumor Fraction	Genomic Coverage	# Libraries	# read pairs (2 x 150 bp)
SVs	> 50%	10X	1	100M
SVs, SNVs, Indels, CNVs	> 50%	30X	1	300M
SVs	20% - 50%	30X	1	300M
SVs, SNVs, Indels, CNVs	20% - 50%	80X	3	800M

Detection of Low VAF events are dependent on sequence depth.



## Appendix 1: Dual Index Primers

LinkPrep™ 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.**

NOTE LinkPrep 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 with a unique I7 Index Primer in steps 2 and 3 of Stage 3.5 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

NOTE Refer to the first page in the following Illumina Guide for how to enter the i5 in forward or reverse complement orientation on the **sample sheet**: <https://support-docs.illumina.com/SHARE/AdapterSequences/Content/SHARE/AdapterSeq/Overview.htm#Index>.

i5 Index Name	Bases in Adapter	Bases for Sample Sheet in Forward Orientation	Bases for Sample Sheet in Reverse Complement Orientation
I5 Index Primer 1	GCGTAAGA	GCGTAAGA	TCTTACGC
I5 Index Primer 2	CTCTCTAT	CTCTCTAT	ATAGAGAG
I5 Index Primer 3	TATCCTCT	TATCCTCT	AGAGGATA
I5 Index Primer 4	CGTCTAAT	CGTCTAAT	ATTAGACG

i7 Index Name	Bases in Adapter	Bases for Sample Sheet
I7 Index Primer 1	TCGCCTTA	TAAGGCGA
I7 Index Primer 2	CTAGTACG	CGTACTAG
I7 Index Primer 3	TTCTGCCT	AGGCAGAA
I7 Index Primer 4	CAGCCTCG	CGAGGCTG
I7 Index Primer 5	AGGAGTCC	GGACTCCT
I7 Index Primer 6	CATGCCTA	TAGGCATG

## Appendix 2: Troubleshooting Guide For Yields

- This guide enables users to troubleshoot:
  - o Purified DNA yield (end of Stage 2) < 150 ng.
  - o Library yield (end of Stage 3) < 200 ng.

### Troubleshooting Purified DNA Yield (End of Stage 2) < 150 ng

**Reason:** Insufficient input material (used less than recommended starting amount or chromatin content in the sample is low).

**Recommendation:** Perform the Lysate QC (Appendix 3) to quantify the chromatin content recovered from the sample and check the fragmentation size distribution.

Fragmentation profile <i>within</i> recommended range and lysate yield < 300 ng	Fragmentation profile <i>outside</i> recommended range and lysate yield < 300 ng
Redo the sample preparation with <b>double</b> the input amount.	Refer to Appendix 4: Troubleshooting Guide For Fragmentation Profile and redo the sample preparation with <b>double</b> the input amount.
<p>NOTE</p> <ul style="list-style-type: none"> <li>• You may choose to combine the purified DNA at the end of Stage 2 for library conversion at Stage 3 (refer to Appendix 5 for how to combine samples for library preparation).</li> <li>• You may choose to produce two technical replicate libraries to pool for deep sequencing to 300M read pairs.</li> </ul>	

### Troubleshooting Library Yield (End of Stage 3) < 200 ng

Reason	Recommendation
1. You started the library preparation with < 150 ng.	1. Shallow sequence the library and run a QC analysis to assess long-range and complexity. If needed, generate another library to pool for deep sequencing.
2. The chromatin was not properly fragmented.	2. If the sample was under-fragmented, insufficient proximity ligated molecules are available for library conversion. Perform Lysate QC (Appendix 3) and check the fragment size distribution. Refer to Troubleshooting Guide for under-fragmented samples to improve the fragmentation profile.
3. Low proximity ligation efficiency.	3. Beside a user error during the preparation, impurities found in some samples can lower the efficiency of the reactions. Filtering the lysate at the end of Stage 1 through a MiniStrainer before proceeding with proximity ligation can help.

## Appendix 3: Lysate QC

- This guide enables users to troubleshoot unexpected yields at Stage 2 or Stage 3 by:
  - o Quantifying the lysate yield recovered at the end of sample preparation (Stage 1).
  - o Confirming that the chromatin was properly fragmented in Stage 1.
- The QC 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

### Before You Begin

- 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 prior to use.
- Equilibrate the Chromatin Capture Beads to room temperature.

### Follow the steps below for Lysate QC:

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Thaw the QC tube that was stored at -80°C (see Note at the end of Stage 1). Pipet mix the 2.5 µL aliquot.
3. Transfer 50 µL of the resuspended Chromatin Capture Beads to the QC tube containing the 2.5 µL aliquot.
4. Pipet up and down to fully mix. Incubate the QC tube at room temperature, off the magnetic rack, for 10 minutes.
5. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Discard the supernatant.
6. 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.
7. Repeat step 6 once, for a total of 2 washes.
8. After removing the last wash, add to the QC tube 51.5 µL of a master mix containing the following reagents **added in the order listed**:

Reagent	Volume Per Reaction
Crosslink Reversal Buffer	50 µL
Proteinase K	1.5 µL
<b>Total</b>	<b>51.5 µL</b>

9. Pipet up and down to fully mix. Incubate the tube in an agitating thermal mixer set at 1,200 rpm as follows:

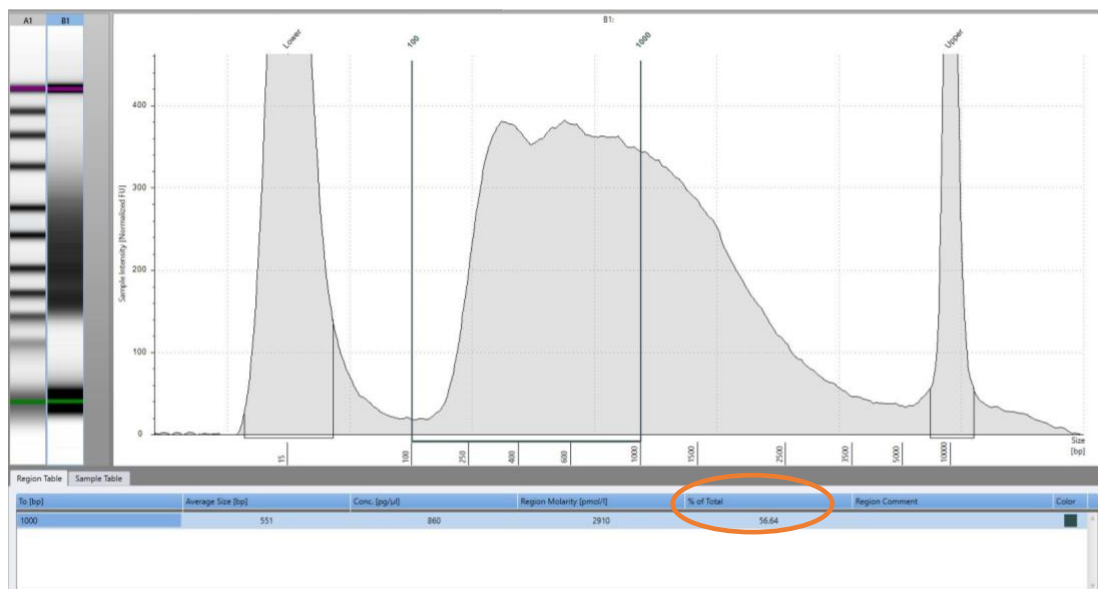
Temperature	Time
78°C	10 minutes
25°C	Hold
Note: secure the tube lid to prevent opening during incubation	

10. Quick spin the tube and place it on the magnetic rack for 1 minute (or until the solution looks clear). **Transfer the SUPERNATANT** to a new 1.5 mL tube for DNA purification. Discard the beads.
11. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
12. Add 90 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.
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 the tube at room temperature, off the magnetic rack, for 5 minutes.
15. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
16. 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.
17. 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.
18. Air dry the beads for 3 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads.**
19. Off the magnetic rack, resuspend the beads in 10 µL TE Buffer pH 8.0.
20. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
21. Incubate at room temperature, off the magnetic rack, for 2 minutes.
22. Quick spin the tube and place it in the magnetic rack for 1 minute.
23. Transfer 8 µL of the **SUPERNATANT** (purified DNA) to a new tube. Discard the beads.
24. Quantify the purified DNA using a Qubit Fluorometer and Qubit dsDNA HS Kit.
  - a. Based on the Qubit concentration, the total lysate amount (ng) can be calculated as follows:  
Total Lysate (ng) = Qubit reading ng/µL x 10 µL (elution volume) x 23.8 (dilution factor)
  - b. **We expect a yield > 300 ng. Refer to Appendix 2: Troubleshooting Guide For Yields if the yield you obtain is < 300 ng.**
25. 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.
  - The fragmentation profile should **contain the specified DNA in the range of 100 – 1,000 bp described in the Table below.** On the TapeStation System, create a region from 100 – 1,000 bp (see figure 3 below. Creating this region will automatically generate a '% of total' value. This value should be between 40%-75%. **Refer to Appendix 4: Troubleshooting Guide For Fragmentation Profile if the value you obtain is outside of the range recommended for the analysis system you are using.**

NOTE The '% of total' cut-off value varies between analytical instruments. Please refer to the table below for the recommended **fragmentation cut-off value** for the system you are using.

System	DNA in the range of 100 – 1,000 bp (% of total value)
TapeStation	40%-75 %
Bioanalyzer	30%-70%
Fragment Analyzer	60%-85%

Figure 3. Expected (QC Pass) fragmentation profile, as analyzed on HS D5000 ScreenTape



## Appendix 4: Troubleshooting Guide For Fragmentation Profile

- This guide enables users to troubleshoot:
  - o A fragmentation profile that contains **more** than recommended DNA in the 100 – 1,000 bp region (over-fragmented sample).
  - o A fragmentation profile that contains **less** than recommended DNA in the 100 – 1,000 bp region (under-fragmented sample).

### Troubleshooting over-fragmented samples

#### I. Over-fragmented samples that were prepared WITH the Cell Isolation Module:

If the fragmentation profile exceeds the recommended upper limit, restart the sample preparation protocol (Stage 1) and **omit the steps pertaining to Cell Isolation Enzyme Mix** while keeping all the other steps the same. Refer to the table below for guidance on what steps to omit when troubleshooting an over fragmented sample.

Protocol	Sample type	Cell Isolation steps to omit
Stage 1A	Cell lines	Stage 1A: steps 12-14
Stage 1B	Fresh frozen mammalian tissues	Stage 1B: steps 12 - 14
Stage 1C	Cryo-preserved PBMCs	Stage 1C: steps 16 - 18
Stage 1D	Fresh blood ≤ 24 hours post collection	Stage 1D: steps 24-26
Stage 1E	Fresh blood 24 - 72 hours post collection	Stage 1E: steps 18-20

#### II. Over-fragmented mammalian tissues that were prepared WITHOUT the Cell Isolation Module:

If the fragmentation profile exceeds the recommended upper limit, restart the sample preparation (Stage 1B) and use 10.8 µL of 37% formaldehyde instead of 5.4 µL while keeping all the other steps the same. Ensure **fresh** formaldehyde solution is being used for crosslinking. Formaldehyde solution showing white precipitates should not be used.

### Troubleshooting under-fragmented tissue samples

If the profile is under-fragmented (i.e. below the recommended lower limit), re-start the sample preparation (Stage 1B) and incorporate the following two modifications:

1. Perform steps a, b, and c below **before the Nuclei Prep** (i.e. before step 15 in Stage 1B).
  - a. Resuspend the tissue pellet in 105 µL of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

Reagent	Volume Per Reaction
<b>Reconstituted</b> Cell Isolation Enzyme Mix (see Before You Begin in Stage 1B)	100 µL
100 mM CaCl <sub>2</sub>	5 µL
<b>Total</b>	<b>105 µL</b>

- b. Incubate the tube in a thermal mixer **WITHOUT SHAKING** as follows:

Temperature	Time
37°C	<b>30</b> minutes

- c. Spin the tube at 500 x g for 5 minutes in a swinging bucket rotor. Carefully remove and discard the supernatant. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss.

**NOTE To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.**

- d. Proceed to Step 15 in Stage 1B: Fresh frozen mammalian tissue.
2. Filter the sample through the 70 µm MiniStrainer at the end of Stage 1 (after completing Step 23). Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the sample into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The aggregates should be retained in the filter and discarded. You will be proceeding with Stage 2 with the filtrate collected in the tube.

## Appendix 5: Combining Samples for Library Preparation

- This guide enables users to:

- o Concentrate their sample if they are combining purified DNA from end of Stage 2 from two proximity ligation reactions for library conversion.

1. Combine the purified DNA from end of stage 2 from two proximity ligation reactions (total volume 80  $\mu$ L) in a 1.5 mL tube.
2. Vortex the SPRIselect beads thoroughly (> 30 seconds) to resuspend.
3. Add 145  $\mu$ L of resuspended SPRIselect beads to the 1.5 mL tube containing the combined purified DNA sample.
4. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
5. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
6. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
7. Leave the tube in the magnetic rack and wash the beads **twice** with 200  $\mu$ L **fresh** 80% ethanol. Do not resuspend the beads for these washes. Add the ethanol, wait for 1 minute then discard the ethanol supernatant.
8. After the last wash, quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10  $\mu$ L pipet tip to remove traces of ethanol.
9. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. Do not over dry the beads.
10. Off the magnetic rack, resuspend the beads in 42  $\mu$ L TE Buffer pH 8.0.
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 at room temperature, off the magnetic rack, for 5 minutes.
13. Quick spin the tube and place it in the magnetic rack for 1 minute.
14. Transfer 40  $\mu$ L of the **SUPERNATANT** (purified DNA) to a new tube. Discard the beads.

## Appendix 6: 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<sup>6</sup> cells/mL.

7. Incubate 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  $2 - 10 \times 10^6$  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  $-80^{\circ}\text{C}$  freezer overnight.

**NOTE** An alternative method could be used for rate-controlled cooling to approximately  $-1^{\circ}\text{C}$  /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  $-135^{\circ}\text{C}$ ).