Dovetail® TopoLink™ Kit

USER GUIDE

VERSION 2.0

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Dovetail® TopoLink™ Kit Components and Storage

Each kit contains a sufficient supply of materials to perform 8 reactions. Each kit comes as 4 boxes.

Dovetail® Link Prep 4° C

Components	Size	Storage
10X Wash Buffer	30mL Bottle	2°C to 8°C
Chromatin Capture Beads	2 mL Tube	
Fragmentation Stop	0.5 mL Tube	
Crosslink Reversal Buffer	2 mL Tube	
Nuclei Prep Buffer	2 mL Tube	

Dovetail® Link Prep -20° C

Components	Size	Storage
Fragmentation Enzyme Mix	0.5 mL Tube	-30°C to - 10°C
4X Fragmentation Buffer	0.5 mL Tube	
Proximity Ligation 1 Enzyme Mix	0.5 mL Tube	
5X Proximity Ligation 1 Buffer	0.5 mL Tube	
Proximity Ligation 2 Enzyme Mix	0.5 mL Tube	
5X Proximity Ligation 2 Buffer	0.5 mL Tube	
Proximity Ligation 3 Enzyme Mix	0.5 mL Tube	
5X Proximity Ligation 3 Buffer	0.5 mL Tube	
Proteinase K	0.5 mL Tube	

Dovetail® TopoLink Library Module for Illumina

Components	Size	Storage
Library Prep 1 Enzyme Mix	0.5 mL Tube	-30°C to - 10°C
5X Library Prep 1 Buffer	0.5 mL Tube	
Library Prep 2 Enzyme Mix	0.5 mL Tube	
2X Library Prep 2 Buffer	0.5 mL Tube	
Library Prep 3 Enzyme Mix	0.5 mL Tube	
HotStart PCR Ready Mix	0.5 mL Tube	

Dovetail® TopoLink™ Primer Module

Components	Size	Storage
I5 Index Primer 1	0.5 mL Tube	-30°C to - 10°C
I5 Index Primer 2		
I5 Index Primer 3		
I5 Index Primer 4		
I7 Index Primer 1		
I7 Index Primer 2		
I7 Index Primer 3		
I7 Index Primer 4		
I7 Index Primer 5		
I7 Index Primer 6		

Dovetail® Cell Isolation Module

Components	Size	Storage
Cell Isolation Enzyme Mix (powder)	2 mL Tube	2°C to 8°C*
Reconstitution Buffer	2 mL Tube	
100mM $CaCl_2$	0.5 mL Tube	

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

User Supplied Reagents, Consumables and Equipment

Reagents

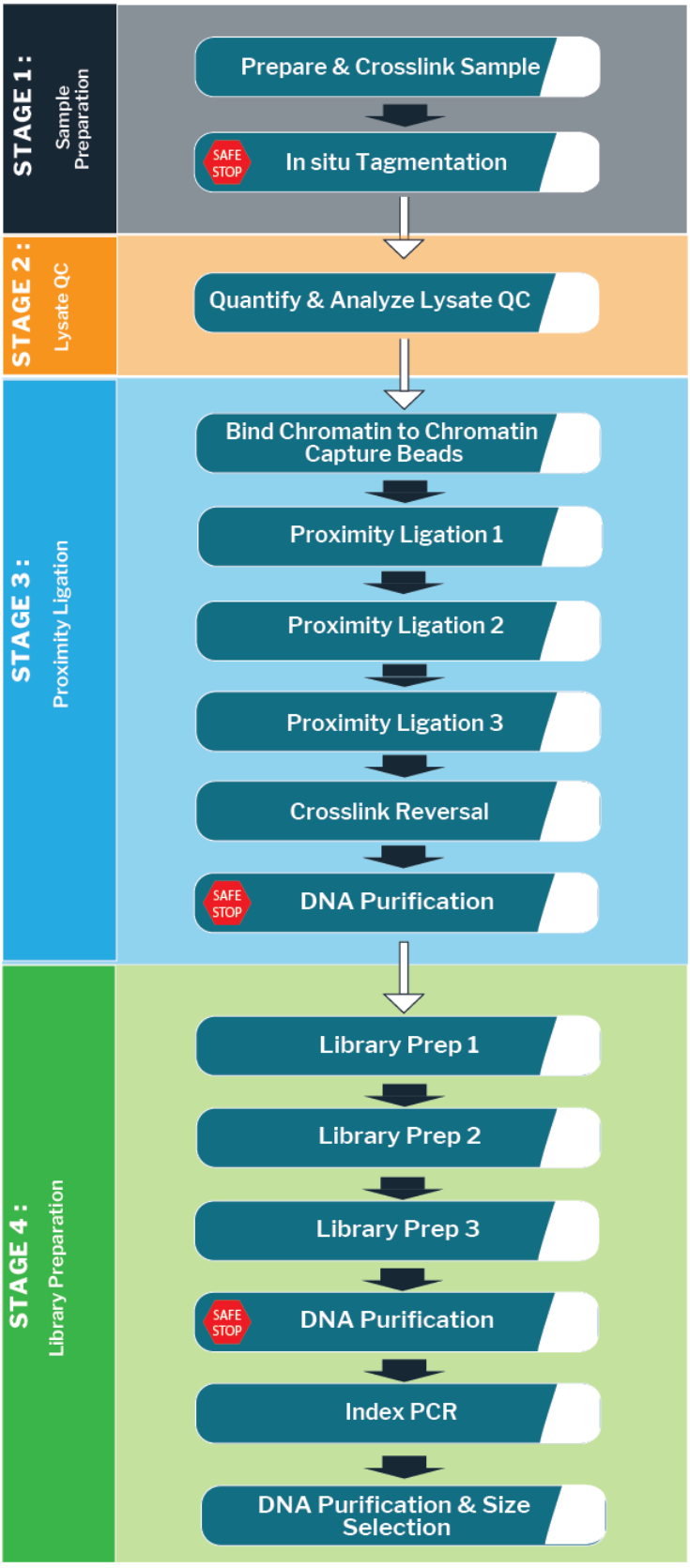
Reagent	Supplier	Part Number
SPRIselect® Beads, 5 mL	Beckman Coulter	B23317
37% Formaldehyde Solution	Sigma-Aldrich	F8775
1X PBS, pH 7.4, 500 mL	Thermo Fisher Scientific	10010023
100% EtOH	Generic	N/A
UltraPure™ DNase / RNase-Free Distilled Water, 500 mL	Thermo Fisher Scientific	10977015
DSG (Disuccinimidyl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

Consumables & Equipment

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 bucker 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® TopoLink 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 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 - 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.

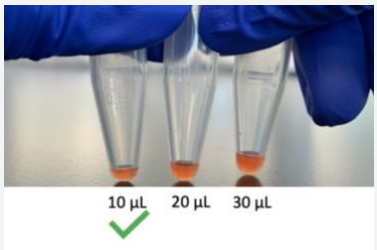
Follow the steps below for Sample Preparation:

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot 1 x 10^6 cells into a 1.5 mL tube.
3. Spin the 1 x 10^6 cell aliquot 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**. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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.
- Minimizing cell loss during sample preparation is important to the success of the assay. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette. Leave behind up to 10 µL to not disturb the pellet (figure 1).**

Figure 1



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

4. Resuspend the cell pellet in 50 µL of 1X PBS and pipet up and down to ensure no clumps are present then add 150 µL of 1X PBS to bring the volume to 200 µL. Pipet up and down to fully resuspend the pellet.
5. Add 2 µL 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 µL 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. Use caution, the pellet might be loose. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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

10. Wash the pellet with 150 µL 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. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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 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 $CaCl_2$	2.5 µL
Total	**52.5 µL**

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. 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.**

15. Resuspend the cells in 100 µL 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. Be careful to not disturb the pellet (which may be hard to visualize), leave behind up to ~10 µL (see figure 1).

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
Total	**52.5 µL**

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. Proceed to Lysate QC (Stage 2) to ensure the fragmentation is successful and to determine the volume of lysate to bind for Proximity Ligation (Stage 3). The fragmentation profile is predictive of library quality, as such the Lysate QC step provides clear go/no go metric. It enables users to take corrective measure early on in the protocol, if needed.

Stage 1B: 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.

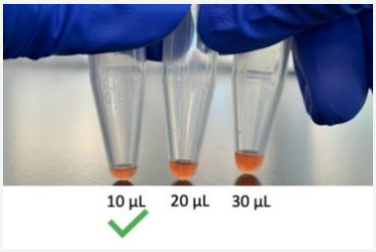
NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be 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 x 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.
- Minimizing cell loss during sample preparation is important to the success of the assay. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette. Leave behind up to 10 µL to not disturb the pellet (figure 1).**

Figure 1



- 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 2). 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. Use caution, the pellet might be loose. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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 $CaCl_2$	2.5 µL
Total	**52.5 µL**

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. 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.**

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. Be careful to not disturb the pellet (which may be hard to visualize), leave behind up to ~10 µL(see figure 1).

> 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
Total	**52.5 µL**

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. Proceed to Lysate QC (Stage 2) to ensure the fragmentation is successful and to determine the volume of lysate to bind for Proximity Ligation (Stage 3). The fragmentation profile is predictive of library quality, as such the Lysate QC step provides clear go/no go metric. It enables users to take corrective measure early on in the protocol, if needed.

Stage 1C: Sample Preparation - Fresh blood ≤ 24h post collection

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

- Blood sample preparation takes ~ 2 hours.

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 10^6 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.
8. Pour the top layer containing the PBMCs into a new 15 mL tube.

   N O T E Do not hold the tube in the inverted position for longer than 2 seconds.
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.

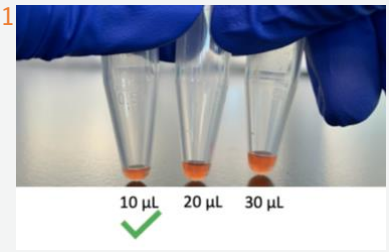
> NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should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

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.
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.**
15. Gently, pipette mix the PBMCs on ice. Transfer 1 x 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.
- Minimizing cell loss during sample preparation is important to the success of the assay. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette. Leave behind up to 10 µL to not disturb the pellet (figure 1).**

Figure 1



- All crosslinking reactions (steps 17 – 23) 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 2). Otherwise, proceed with the protocol.

16. Bring up the volume to 200 µL of 1X PBS, if needed.
17. Add 2 µL of 0.3 M DSG. Pipet to mix.
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 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**. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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

22. Wash the pellet with 150 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. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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 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 $CaCl_2$	2.5 µL
Total	**52.5 µL**

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. 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.**

27. Resuspend the cells in 100 µL 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. Be careful to not disturb the pellet (which may be hard to visualize), 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.**

30. 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
Total	**52.5 µL**

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 µL 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 6µL 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. Proceed to Lysate QC (Stage 2) to ensure the fragmentation is successful and to determine the volume of lysate to bind for Proximity Ligation (Stage 3). The fragmentation profile is predictive of the library quality, as such the Lysate QC step provides clear go/no go metric. It enables users to take corrective measure early on in the protocol, if needed.

Stage 1D: Sample Preparation - Fresh blood 24-72h post collection

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

- Blood sample preparation takes ~ 2 hours.

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 effect cell counting accuracy.
  - >> Warm eBioscience™ 10X RBC Lysis Buffer to room temperature (15 - 25°C) before use.
  - >> Typically, 0.5 to 3 x 10^6 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 3mL 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.

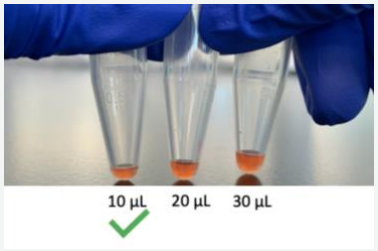
NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be 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 x 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.
- Minimizing cell loss during sample preparation is important to the success of the assay. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette. Leave behind up to 10 µL to not disturb the pellet (figure 1).**

Figure 1



- 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 2). 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. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette.** Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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 µL
100mM $CaCl_2$	2.5 µL
Total	**52.5 µL**

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. 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.**

21. Resuspend the cells in 100 µL 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. Be careful to not disturb the pellet (which may be hard to visualize), 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.**

24. 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
Total	**52.5 µL**

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 µL 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 6µL 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. Proceed to Lysate QC (Stage 2) to ensure the fragmentation is successful and to determine the volume of lysate to bind for Proximity Ligation (Stage 3). The fragmentation profile is predictive of the library quality, as such the Lysate QC step provides clear go/no go metric. It enables users to take corrective measure early on in the protocol, if needed.

Stage 1E: 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

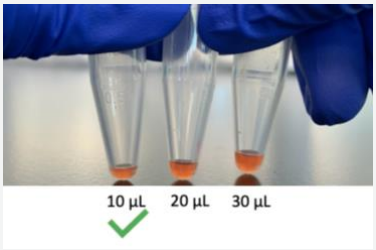
- You may choose to freeze your ground/disrupted tissue pellet at -80°C at this stage. Otherwise, proceed with the protocol.
- All crosslinking reactions (steps 5 – 11) should be carried out at room temperature.

4. Resuspend the tissue pellet in 200 µL of 1X PBS and pipet up and down to ensure no clumps are present.
5. Add 2 µL 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 µL 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. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette**. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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.
- Minimizing cell loss during sample preparation is important to the success of the assay. **To minimize cell loss, remove the supernatant SLOWLY from the TOP DOWN, using a P200 pipette. Leave behind ~10 µL to not disturb the pellet (figure 1).**

Figure 1



10. Wash the tissue pellet with 100 µL 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. Be careful to not disturb the pellet, leave behind ~10 µL to avoid cell loss (see figure 1).

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 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)	100 µL
100mM $CaCl_2$	5 µL
Total	**105 µL**

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

Temperature	Time
37°C	**30** minutes

14. 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.**

15. Resuspend the pellet in 100 µL 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. Be careful to not disturb the pellet (which may be hard to visualize), 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.**

18. 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
Total	**52.5 µL**

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 6µL 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. **For muscular tissues** (such as quadricep, gastrocnemius, tibialis, heart, tail) 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 25.**
25. Proceed to Lysate QC (Stage 2) to ensure the fragmentation is successful and to determine the volume of lysate to bind for Proximity Ligation (Stage 3). The fragmentation profile is predictive of the library quality, as such the Lysate QC step provides clear go/no go metric. It enables users to take corrective measure early on in the protocol, if needed.

Stage 2: Lysate QC

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

- The Lysate QC stage takes ~ 1 hour.
- This stage has 2 objectives:
    - Confirm sufficient lysate is available to use in proximity ligation (Stage 3).
    - Determine the volume of lysate to use in proximity ligation (Stage 3).
    - Confirm that the chromatin was fragmented.
- 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

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. Transfer 50 µL of the resuspended Chromatin Capture Beads to a new 1.5 mL tube labeled QC.
3. Pipet mix the lysate then add 2.5 µL of the lysate to the 1.5 mL QC tube containing the beads.

> NOTE **Place the remaining lysate on ice. This is the lysate you will be using in Stage 3. If you don't plan to proceed with Stage 3 on the same day, store the remaining lysate at -80°C.**

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
Total	**51.5 µL**

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 Note: secure the tube lid to prevent opening during incubation	Hold

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. Proceed to Stage 3 with a volume of your lysate that corresponds to no more than 3,000 ng (**do not exceed 3,000 ng**). This volume can be calculated using the following equation:

$$volume\ (\mu L) = \frac{3000\ ng\ x\ 59.5\ \mu L}{total\ lysate\ (ng)}$$

    If your sample has ≤ 3,000 ng proceed using the entire volume for binding in Stage 3.
    c. For 1 x 10^6 cells, we expect the DNA yield to be in the range of 1,000 – 3,000 ng. Lower lysate yield may result in insufficient DNA for a high complexity library at Stage 4. If you recover less than 500 ng, we don't recommend you proceed. Contact support@cantatabio.com for recommendations.
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.

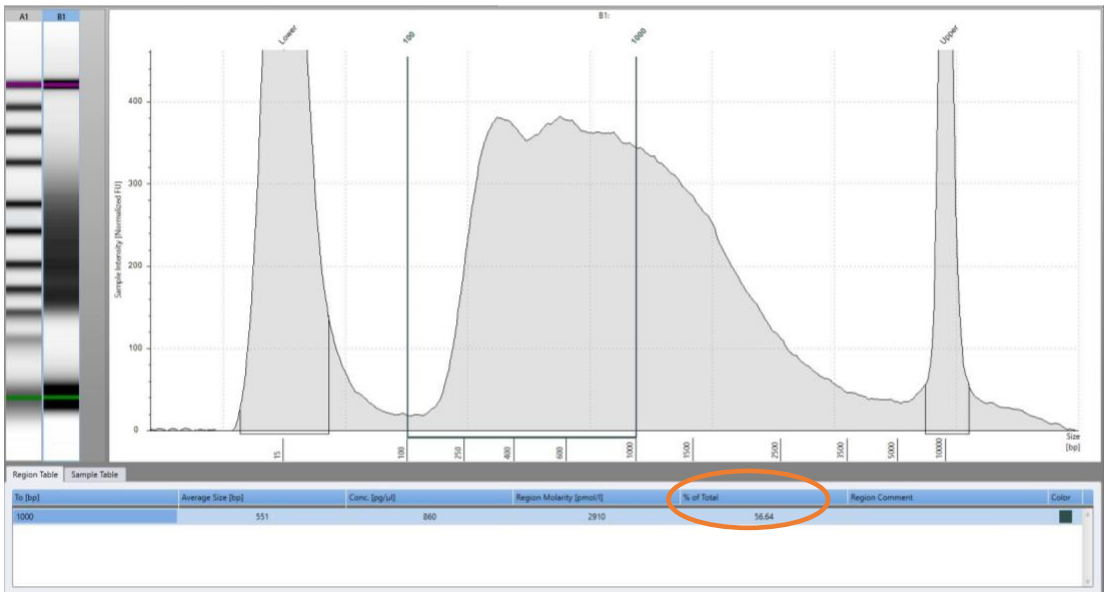
- The fragmentation profile should **contain the specified DNA in the range of 100 – 1,000 bp described in the Table below.** On the TapeStation System, create a region from 100 – 1,000 bp (see figure 2 below). Creating this region will automatically generate a '% of total' value. This value should be between 40%-75%.

N O T E 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%

- If the fragmentation profile contains **less** than recommended DNA in the 100 – 1,000 bp region, the library complexity may be reduced (i.e. the library will have a higher duplication rate). Please contact support@cantatabio.com to help troubleshoot an under fragmented profile.
- If the fragmentation profile contains **more** than recommended DNA in the 100 – 1,000 bp region, the library may contain lower fraction cis long-range read pairs. You have 2 options: proceed to Stage 3 knowing that the final library may contain lower cis long-range information **or** restart the protocol and refer to Appendix 3: Troubleshooting Guide for Over Fragmented Samples.

Figure 2. Expected (QC Pass) fragmentation profile, as analyzed on HS D5000 ScreenTape



Stage 3: Proximity Ligation

As you prepare for Stage 3, 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.

3.1 Bind Chromatin to Chromatin Capture Beads

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

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Transfer 100 µL of resuspended Chromatin Capture Beads to a **new** 0.2 mL PCR tube.
3. Add up to 3,000 ng equivalent of your lysate (see step3 NOTE in Stage 2) to the Chromatin Capture Beads aliquoted above. If you have recovered >3,000 ng, you can store the remaining lysate at -80°C.

> NOTE See calculations in Stage 2, step 24 to determine the volume equivalent to 3,000 ng.

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 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. Remove the tube from the magnetic rack and resuspend the beads in 150 µL 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.**

3.2 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 µL
5X Proximity Ligation 1 Buffer	10 µL
Proximity Ligation 1 Enzyme Mix	2.5 µL
Total	**52.5 µL**

2. Place the tube from step 7 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 52.5 µL of the proximity ligation 1 master mix.

4. 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

5. Quick spin the tube and place the tube 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 fully resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

3.3 Proximity Ligation 2

Follow the steps below for Proximity Ligation 2:

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

Reagent	Volume Per Reaction
UltraPure Water	40 µL
5X Proximity Ligation 2 Buffer	10 µL
Proximity Ligation 2 Enzyme Mix	5 µL
Total	**55 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 55 µL of the proximity ligation 2 master mix.
4. 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

5. Quick spin the tube and place the tube 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 fully resuspend the beads. **Leave the tube on the bench while you prepare for the next reaction.**

3.4 Proximity Ligation 3

Follow the steps below for Proximity Ligation 3:

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

Reagent	Volume Per Reaction
UltraPure Water	40 µL
5X Proximity Ligation 3 Buffer	10 µL
Proximity Ligation 3 Enzyme Mix	1 µL
Total	**51 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 51 µL of the proximity ligation 3 master mix.

4. 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

5. Quick spin the tube and place the tube 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 **added in the order listed**:

Reagent	Volume Per Reaction
Crosslink Reversal Buffer	50 µL
Proteinase K	1.5 µL
Total	**51.5 µL**

2. 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 Note: secure the tube lid to prevent opening during incubation.	Hold

3. 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.

3.6 DNA Purification

Follow the steps below for DNA Purification:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 90 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 42 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 2 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. **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 in the range of 500 ng – 2,000 ng.

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 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 TopoLink Dual Index Primer Set at room temperature. Leave on ice once thawed. Mix by inversion and quick spin prior to use.

NOTES

- The TopoLink kit supports conversion of 3 libraries from 1 proximity ligation reaction (i.e. from the DNA you recover at the end of Stage 3) to enable loop calling applications. To maximize complexity of each library, we recommend you split the DNA from step 13 Stage 3.6 equally between the 3 library conversions with a **minimum input of 250ng *per* library prep**.
- 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.

4.1 Library Prep 1

Follow the steps below for Library Prep 1:

1. Place **≥ 250 ng of purified DNA** from step 13 Stage 3.6 in a new 0.2 mL PCR tube and bring the volume to 40 µL with UltraPure Water.
2. Add to the tube the following reagents **in the order listed**:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
5X Library Prep 1 Buffer	10 µL	11 µL	x	3	=	33 µL
Library Prep 1 Enzyme Mix	1.5 µL	1.65 µL	x	3	=	4.95 µL
Total	**11.5 µL**					

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

4.2 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	10% Extra		# Reactions		Final
2X Library Prep 2 Buffer	50 µL	55	x	3	=	165 µL
Library Prep 2 Enzyme Mix	1 µL	1.1	x	3	=	3.3 µL
Total	**51 µL**					

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

4.3 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

4.4 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. **Transfe**r 15 µL of the **SUPERNATANT** (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.

4.5 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 4.4.
2. Add 5 µL of TopoLink I5 Index Primer to the PCR reaction (see Appendix 1: Dual Index Primers).
3. Add 5 µL of TopoLink I7 Index Primer to the PCR reaction (see Appendix 1: Dual Index Primers).

4. 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:

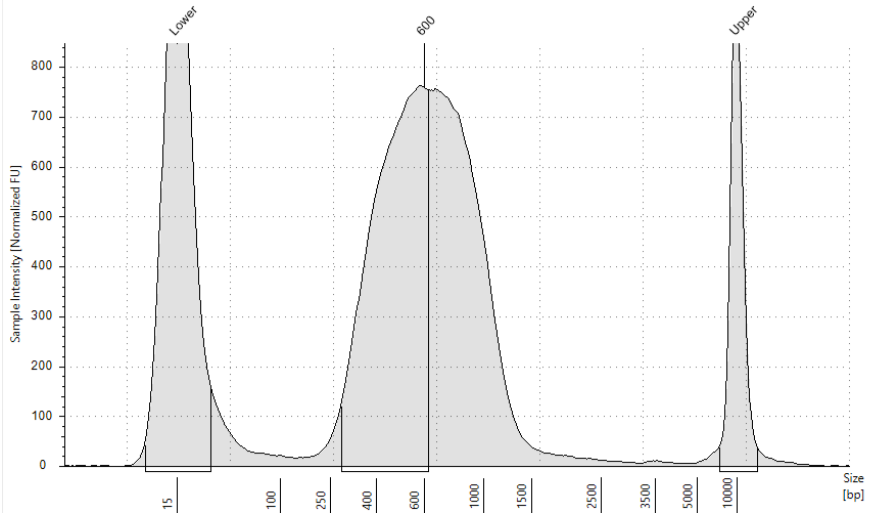
Step	Temperature	Time	Cycles
Enzyme Activation	98°C	3 minutes	1
Denature	98°C	20 seconds	
Anneal	60°C	30 seconds	12
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	

4.6 Size Selection

Follow the steps below for Size Selection:

1. 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.
2. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
3. Add 50 µL of resuspended SPRIselect beads to the tube containing your 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 5 minutes.
6. Quick spin the tube and place it in the magnetic rack for 5 minutes.
7. **Transfer 145 µL of the SUPERNATANT** to a new 0.2 mL PCR tube. Discard the beads.
8. Add 30 µL of resuspended SPRIselect beads to the 0.2 mL PCR tube containing your sample.
9. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
10. Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
11. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
12. 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.
13. 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.
14. Air dry the beads for 3 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads.**
15. Off the magnetic rack, resuspend the beads in 32 µL TE Buffer pH 8.0.
16. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
17. Incubate the tube at room temperature, off the magnetic rack, for 2 minutes.
18. Quick spin the tube and place it in the magnetic rack for 1 minute.
19. **Transfer** 30 µL of the **SUPERNATANT** to a new 0.2 mL PCR tube. The supernatant is your size selected library. Discard the beads.
20. Quantify your size selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit. We expect a final library yield in the range of 500 ng – 1500 ng.
21. 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 3).

Figure 3. 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

We recommend that you sequence your library to ~10 M read pairs (2 x 150 bp) to run a QC analysis prior to deep sequencing. Please contact support@cantatabio.com for recommendations regarding the QC analysis and sequencing depth.

Appendix 1: Dual Index Primers

TopoLink™ Dual Index Primer Module includes four I5 index primers and six I7 index primers.
Verify that the indexes selected for pooling have the appropriate color balance.

NOTE The TopoLink™ Dual Index Primer Module 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 4.5 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

Primer name	i5 index (HiSeq® 2000/2500, MiSeq®, NovaSeq® Illumina® systems)	i5 index (HiSeq® 3000, 4000, X, NextSeq®, MiniSeq®, iSeq® Illumina® systems)
I5 Index Primer 1	GCGTAAGA	TCTTACGC
I5 Index Primer 2	CTCTCTAT	ATAGAGAG
I5 Index Primer 3	TATCCTCT	AGAGGATA
I5 Index Primer 4	CGTCTAAT	ATTAGACG

Primer name	i7 index
I7 Index Primer 1	TAAGGCGA
I7 Index Primer 2	CGTACTAG
I7 Index Primer 3	AGGCAGAA
I7 Index Primer 4	CGAGGCTG
I7 Index Primer 5	GGACTCCT
I7 Index Primer 6	TAGGCATG

Appendix 2: 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 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).

Appendix 3: Troubleshooting Guide for Over Fragmented Samples

Refer to this troubleshooting guide if the fragmentation profile for your lysate QC (Stage 2) exceeds the recommended upper limit for fragmentation.

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

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	Cryo-preserved PBMCs	Stage 1B: steps 16-18
Stage 1C	Fresh blood ≤ 24 hours post collection	Stage 1C: steps 24-26
Stage 1D	Fresh blood 24-72 hours post collection	Stage 1D: steps 18-20
Stage 1E	Fresh frozen mammalian tissues	Stage 1E: steps 12-14