



# Dovetail® AssemblyLink™ Kit USER GUIDE

Version 1.0  
May 30, 2024

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**For technical customer support, please contact**

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

+1 (831) 233-3780

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## Dovetail® AssemblyLink Kit Components and Storage

Each kit contains a sufficient supply of materials to perform 8 reactions. Each kit comes as 5 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® AssemblyLink 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® AssemblyLink Primer Module**

Components	Size	Storage
Primer Mix 1	2 mL Tube	-30°C to -10°C
Primer Mix 2		
Primer Mix 3		
Primer Mix 4		
Primer Mix 5		
Primer Mix 6		
Primer Mix 7		
Primer Mix 8		

**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 <sub>2</sub>	0.5 mL Tube	

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

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## 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

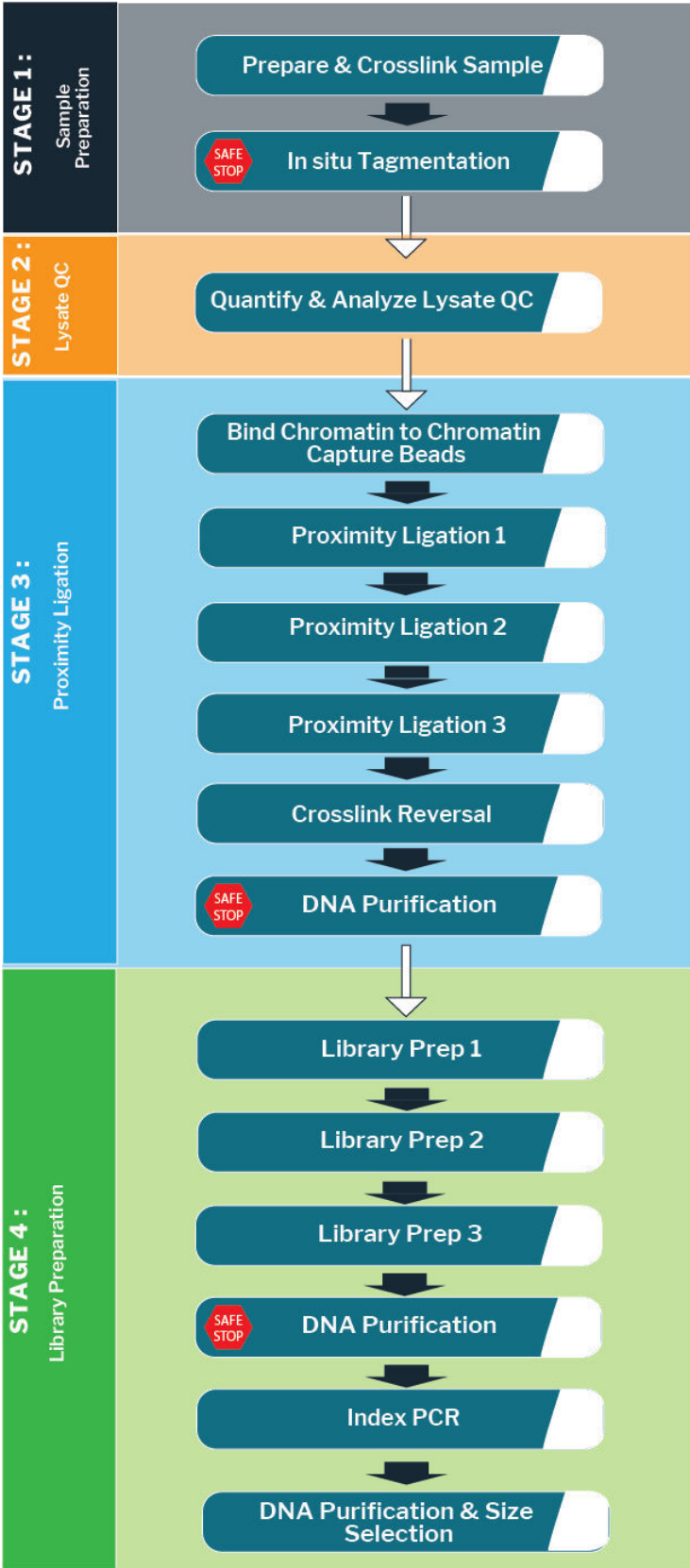
\*only needed if working with Fresh Mammalian Whole Blood (≤ 24 hours post collection)

\*\*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® AssemblyLink 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	Validated non-mammalian samples
GM12878	Blood / PBMCs	Fish
HG002	Liver	Insects
K562	Brain	Marine worms
Detroit562	Lung	Mollusks
HT-1197	Spleen	Cnidarians
LNCaP	Heart	Crustaceans
HCC1187	Quadriceps 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.

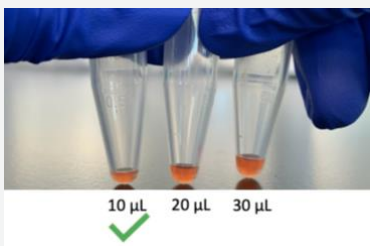
Follow the steps below for Sample Preparation:

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 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  $\mu\text{L}$  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. Use caution, the pellet might be loose. Be careful to not disturb the pellet, leave behind ~10  $\mu\text{L}$  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  $\mu\text{L}$  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. Be careful to not disturb the pellet, leave behind ~10  $\mu\text{L}$  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  $\mu\text{L}$  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. Be careful to not disturb the pellet, leave behind ~10  $\mu\text{L}$  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  $\mu\text{L}$  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. 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
<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. 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 - Fresh frozen animal 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.
- **If working with mammalian tissues**, refer to Appendix 3: Cell Isolation Module before you start the preparation.

Follow the steps below for Sample Preparation:

1. Weigh 10 - 20 mg of frozen tissue.

#### NOTES

- Expected lysate amounts vary between tissues and organisms. For some samples, 5 mg of tissue generates sufficient lysate quantities to proceed through Stage 3.
- If working with mammalian spleen, weigh 3 mg of frozen tissue.
- If working with mammalian muscle, weigh 20 mg of frozen tissue.

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

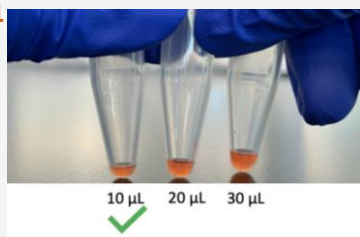
- You may choose to freeze your ground/disrupted tissue pellet at -80°C at this stage. Otherwise, proceed with the protocol.
- All crosslinking reactions (steps 5 – 11) should be carried out at room temperature.

4. Resuspend the tissue pellet in 1 mL of 1X PBS and pipet up and down to ensure no clumps are present.
5. Add 10  $\mu\text{L}$  of 0.3 M DSG. Pipet to mix.
6. Rotate the tube for 10 minutes at room temperature. Sample should not settle.
7. Add 27  $\mu\text{L}$  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. **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  $\mu\text{L}$  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  $\mu\text{L}$  to not disturb the pellet (figure 1).**

**Figure 1**



- If the sample does not seem to be pelleting well, increase the speed to 1,000 x g throughout the preparation.

10. Wash the tissue pellet with 500  $\mu\text{L}$  of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

**NOTE** If you are working with insects, filter your sample through a 70  $\mu\text{m}$  MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the sample (resuspended in Wash Buffer) into the MiniStrainer. Quick spin at 500 x g for 5 seconds. Debris should be retained in the filter and discarded. You will proceed to step 11 with the filtrate collected in the tube.

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  $\mu\text{L}$  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.
- **If you are working with mammalian tissues, refer to Appendix 3 before starting step 12.**

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

Temperature	Time
62°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~~ <sup>supernatant</sup>. 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.

15. 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>

16. 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.

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

Temperature	Time
65°C	5 minutes

19. Quick spin the tube. Add 6µL of 10% Triton. **Pipet up and down to fully mix**.
20. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE**.
21. **For mammalian 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 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 22.
22. 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 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.

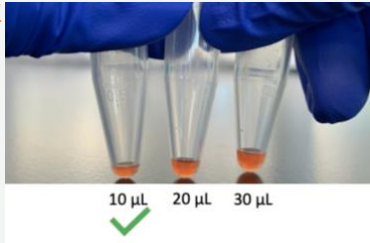
**NOTE** If cell clumping is observed at this stage, filter the sample through the MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be 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.
- 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  $\mu$ L 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  $\mu$ L with 1X PBS.
9. Add 2  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ 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>

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
<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. 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 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.

### 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.
8. Pour the top layer containing the PBMCs into a new 15 mL tube.

**NOTE** Do not hold the tube in the inverted position for longer than 2 seconds.
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  $\mu$ L of 1X Wash Buffer, pipet up and down gently to break up clumps and resuspend the pellet.

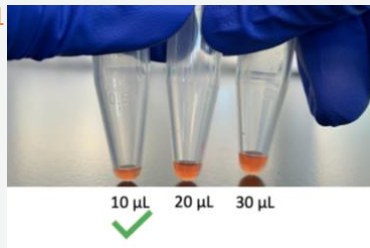
**NOTE** If cell clumping is observed at this stage, filter the sample through the MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be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

12. Centrifuge at 500 x g for 5 minutes. Carefully discard the supernatant.
13. Resuspend the pellet with 200  $\mu$ L 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  $1 \times 10^6$  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.
- 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  $\mu$ L 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  $-80^{\circ}\text{C}$  at this stage (Appendix 2). Otherwise, proceed with the protocol.

16. Bring up the volume to 200  $\mu$ L of 1X PBS, if needed.
17. Add 2  $\mu$ L 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  $\mu$ L 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  $\sim 10$   $\mu$ L 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  $\mu$ 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. 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 <sub>2</sub>	2.5 µL
<b>Total</b>	<b>52.5 µL</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. 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
<b>Total</b>	<b>52.5 µL</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 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 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.

### 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<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 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.

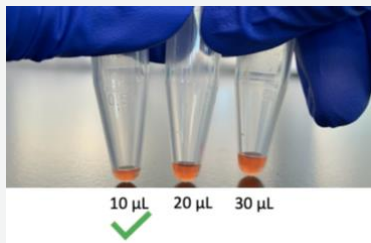
**NOTE** If cell clumping is observed at this stage, filter the sample through the MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be 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  $\mu$ L 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.
- 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  $\mu$ L 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  $\mu$ L of 1X PBS, if needed.
11. Add 2  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ L 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  $\mu$ 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>

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  $\mu$ L 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  $\mu$ 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. Be careful to not disturb the pellet (which may be hard to visualize), leave behind ~10  $\mu$ L 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  $\mu$ 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 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 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:
  - o Confirm sufficient lysate is available to use in proximity ligation (Stage 3).
  - o Determine the volume of lysate to use in proximity ligation (Stage 3).
  - o 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
<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.
- 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)
  - Proceed to Stage 3 with a volume of your lysate that corresponds to up to 2,000 ng (**do not exceed 2,000 ng**). This volume can be calculated using the following equation:
$$volume\ (\mu L) = \frac{2000\ ng \times 59.5\ \mu L}{total\ lysate\ (ng)}$$

If your sample has  $\leq 2,000$  ng proceed using the entire volume for binding in Stage 3.
  - If you recover less than 300 ng, contact [support@cantatabio.com](mailto: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.

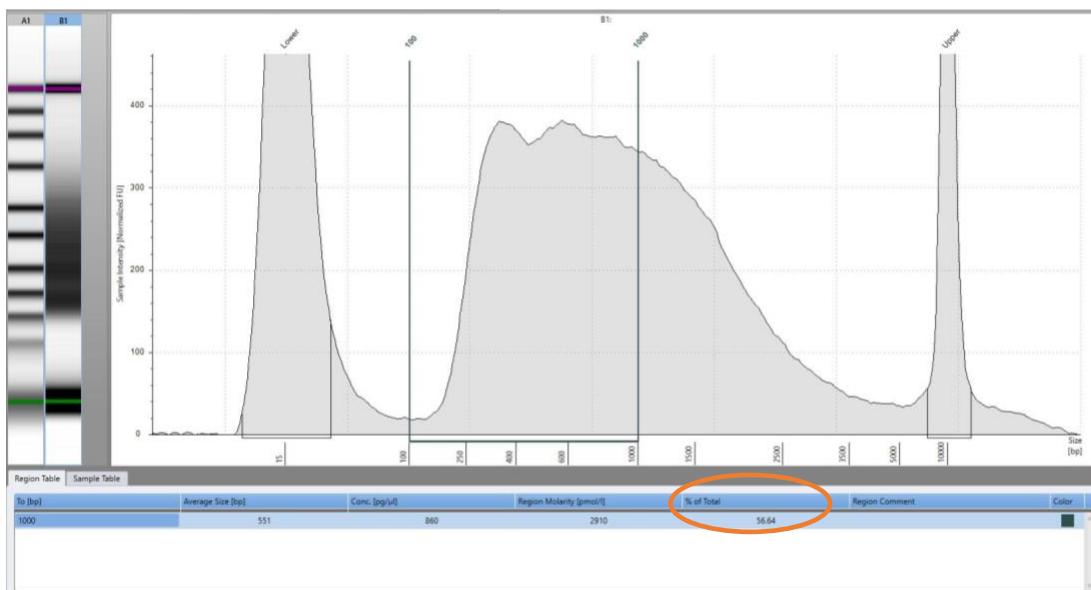
- The fragmentation profile should **contain the specified DNA in the range of 100 – 1,000 bp described in the Table below**. On the TapeStation System, create a region from 100 – 1,000 bp (see figure 2 below). Creating this region will automatically generate a '% of total' value. This value should be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

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%

- 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](mailto: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 2: 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 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 µL of resuspended Chromatin Capture Beads to a **new** 0.2 mL PCR tube.
3. Add up to 2,000 ng equivalent of your lysate (see step3 NOTE in Stage 2) to the Chromatin Capture Beads aliquoted above. If you have recovered >2,000 ng, you can store the remaining lysate at -80°C.

NOTE See calculations in Stage 2, step 24 to determine the volume equivalent to 2,000 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
<b>Total</b>	<b>52.5 µL</b>

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.

- 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.**

### 3.3 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 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**).
- 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.**

### 3.4 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 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**).
  - 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.

### 3.5 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.

### 3.6 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.

**SAFE STOP** Purified DNA sample can be stored at  $-20^{\circ}\text{C}$  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 AssemblyLink Dual Index Primer Set 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.

### 4.1 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 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
5X Library Prep 1 Buffer	10 µL
Library Prep 1 Enzyme Mix	1.5 µL
<b>Total</b>	<b>11.5 µL</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

### 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
2X Library Prep 2 Buffer	50 µL
Library Prep 2 Enzyme Mix	1 µL
<b>Total</b>	<b>51 µL</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

### 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

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.

### 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. **Transfer** 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 10 µL of AssemblyLink Dual Index Primer Mix to the PCR reaction. Use one Primer Mix per 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:

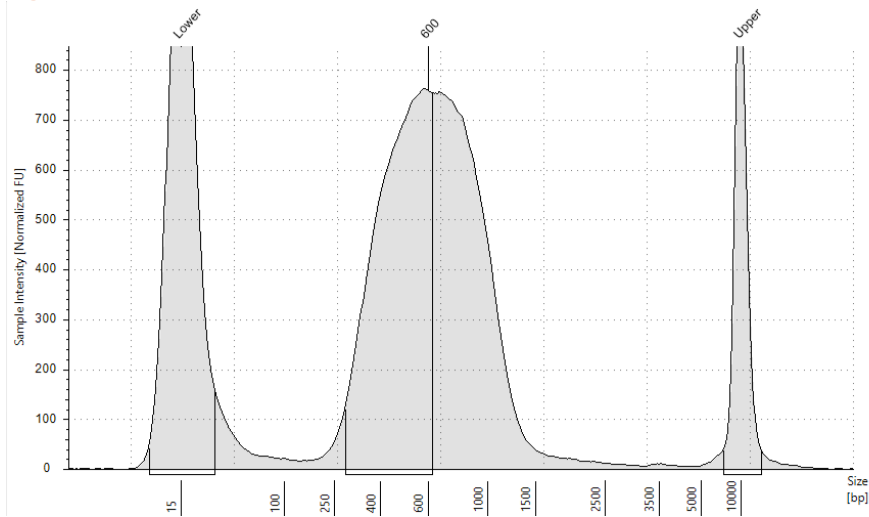
Step	Temperature	Time	Cycles
Enzyme Activation	98°C	3 minutes	1
Denature	98°C	20 seconds	12
Anneal	60°C	30 seconds	
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	

## 4.6 Size Selection

Follow the steps below for Size Selection:

- 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.**
- 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

Dovetail® AssemblyLink™ libraries are sequenced via Illumina® sequencers in paired-end mode. Each library can be deep sequenced to 300M read pairs (2 x 150 bp). We recommend shallow sequencing the library to run a QC analysis prior to deep sequencing. 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://assemblylink.readthedocs.io/en/latest/>).

## Appendix 1: Dual Index Primers

AssemblyLink Dual Index Primer Module includes the following eight Primer Mixes. **Verify that the indexes selected for pooling have the appropriate color balance.**

Primer name	i5 index (HiSeq® 2000/2500, MiSeq®, NovaSeq® Illumina® systems)	i5 index (HiSeq® 3000, 4000, X, NextSeq®, MiniSeq®, iSeq® Illumina® systems)	i7 index
Primer Mix 1	GCGTAAGA	TCTTACGC	TAAGGCGA
Primer Mix 2	CTCTCTAT	ATAGAGAG	CGTACTAG
Primer Mix 3	TATCCTCT	AGAGGATA	AGGCAGAA
Primer Mix 4	CGTCTAAT	ATTAGACG	CGAGGCTG
Primer Mix 5	GTAAGGAG	CTCCTTAC	GGACTCCT
Primer Mix 6	ACTGCATA	TATGCAGT	TAGGCATG
Primer Mix 7	AAGGAGTA	TACTCCTT	CTCTCTAC
Primer Mix 8	CTAAGCCT	AGGCTTAG	CAGAGAGG

## Appendix 2: Troubleshooting Guide for Over or Under Fragmented Samples

Refer to this troubleshooting guide if the fragmentation profile for your lysate QC (Stage 2) is not within the recommended range for fragmentation.

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

### 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: Appendix 3 steps a, b, c

#### II. Over-fragmented animal 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 54  $\mu$ L of 37% formaldehyde instead of 27  $\mu$ L while keeping all the other steps the same.

### Troubleshooting under-fragmented animal 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:

- Perform the steps a, b, and c below **before the Nuclei Prep** (i.e before step 12 in Stage 1B) .
  - Resuspend the tissue pellet in 105  $\mu$ 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
<b>Reconstituted</b> Cell Isolation Enzyme Mix (see Before You Begin in Stage 1B)	100 $\mu$ L
100 mM CaCl <sub>2</sub>	5 $\mu$ L
<b>Total</b>	<b>105 <math>\mu</math>L</b>

- 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. Be careful to not disturb the pellet, leave behind ~10 to avoid cell loss.

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

- Proceed to Step 12 in Stage 1B: Fresh frozen animal tissue.
- Filter the sample through the 70  $\mu$ m MiniStrainer at the end of Stage 1 ( after completing Step 20). 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 3: Cell Isolation Module

Refer to this appendix if you are working with mammalian tissues. Some mammalian tissues require Cell Isolation Module (see table below). For these tissues, perform the steps a, b, and c below **before the Nuclei Prep** (i.e before step 12 in Stage 1B). For tissues that do not require Cell Isolation Module, follow the standard protocol with no modifications.

Tissue	Cell Isolation Module?
Brain	No
Liver	No
Testes	No
Lung	Yes
Kidney	Yes
Colon	Yes
Spleen	Yes
Heart	Yes
Muscle	Yes

- a. 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
<b>Reconstituted</b> Cell Isolation Enzyme Mix (see Before You Begin in Stage 1B)	100 $\mu\text{L}$
100 mM $\text{CaCl}_2$	5 $\mu\text{L}$
<b>Total</b>	<b>105 <math>\mu\text{L}</math></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 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 12 in Stage 1B: Fresh frozen animal tissue.