
# Dovetail® AssemblyLink™ Kit

## USER GUIDE

Version 2.0

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# Dovetail® AssemblyLink™ Kit Components and Storage

**LinkPrep™ Proximity Core 4°C**

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

**LinkPrep™ Proximity Core -20° C**

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

**LinkPrep™ Library Module for Illumina**

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

**LinkPrep™ Primer Module Set #1**

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

**Dovetail® Cell Isolation Module**

| Components | Volume/Mass | Storage |
|---|---|---|
| Cell Isolation Enzyme Mix (powder) | 45 mg | 2°C to 8°C* |
| Reconstitution Buffer | 1,700 µL | |
| 100mM $CaCl_2$ | 200 µL | |

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

## User Supplied Reagents, Consumables and Equipment

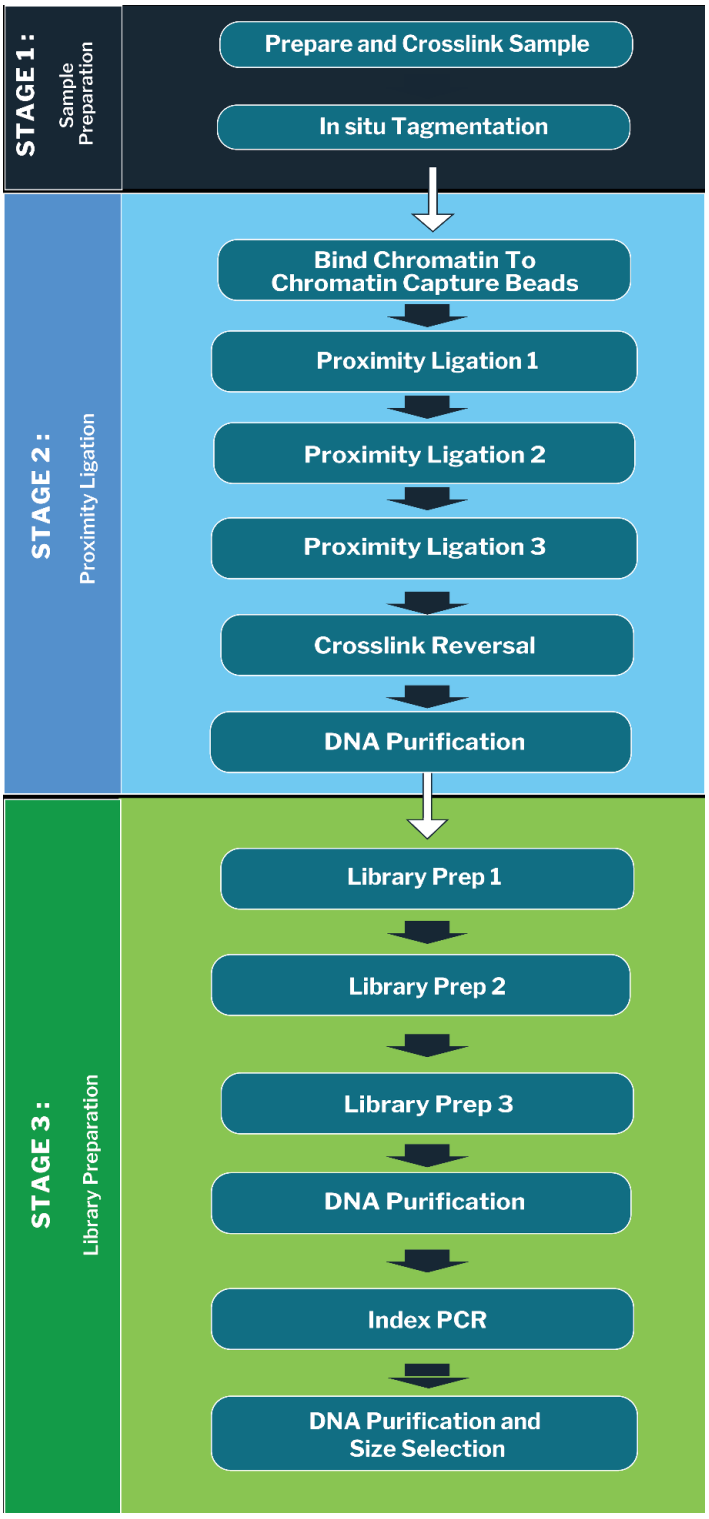
**Reagents**

| Reagent | Supplier | Part Number |
|---|---|---|
| SPRIselect® Beads, 5 mL | Beckman Coulter | B23317 |
| 37% Formaldehyde Solution | Sigma-Aldrich | F8775 |
| 1X PBS, pH 7.4, 500 mL | Thermo Fisher Scientific | 10010023 |
| 100% EtOH | Generic | N/A |
| UltraPure™ DNase / RNase-Free Distilled Water, 500 mL | Thermo Fisher Scientific | 10977015 |
| DSG (Disuccinimidyl Glutarate) | Thermo Fisher Scientific | A35392 |
| DMSO (Dimethyl Sulfoxide, Anhydrous ≥ 99.99%) | Sigma-Aldrich | 276855-100ML |
| TE Buffer pH 8.0 | Thermo Fisher Scientific | AM9849 |
| 10% Triton X-100 | Thermo Fisher Scientific | 85111 |
| SepMate™-15* | Stemcell technologies | 85415 |
| Lymphoprep™* | Stemcell technologies | 07801 |
| eBioscience™ 10X RBC Lysis Buffer** | Thermo Fisher Scientific | 00-4300 |
| CryoStor®*** | BioLife Solutions | 210373 |

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

| Consumable/Equipment | Supplier | Part Number |
|---|---|---|
| 1.5 mL Low binding microcentrifuge tubes | Generic | |
| 0.2 mL PCR tubes | | |
| Pipets and pipet tips | | |
| Magnetic separation rack for 0.2 mL and 1.5 mL tubes | | |
| Agitating thermal mixer | | |
| Thermal cycler | | |
| Vortex mixer | | |
| Centrifuge for 0.2 mL 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® 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 | Quadricep muscle | |
| HEK-293T | Colon | |
| Colo829 | Kidney | |
| NIH-3T3 | Prostate | |
| N2A | Testes | |

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

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

- Sample preparation takes ~ 1 hour and 30 minutes.

Before You Begin

- The 10X Wash Buffer and Fragmentation Stop might have precipitated in storage. **Incubate these solutions at 50°C for 15 minutes** or until the precipitate is no longer visible. Vortex to mix prior to use.
- **Dilute the 10X Wash Buffer to 1X** with UltraPure™ Water. Store at room temperature. 1X Wash Buffer is stable at room temperature for 2 months. You need ~2 mL of 1X Wash Buffer per sample for the entire protocol.
- **Prepare 0.3 M DSG in DMSO** (anhydrous) by dissolving 1 mg of DSG in 10.22 µL DMSO. DSG is water-insoluble and moisture-sensitive. Prepare immediately before use. Do not store DSG in solution.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use**. Make sure there is no precipitate visible. Vortex to mix prior to use.
- **Reconstitute Cell Isolation Enzyme Mix** as follows: on ice, transfer 850 µL of Reconstitution Buffer to the tube containing Cell Isolation Enzyme Mix powder. Pipet up and down to mix. Transfer an additional 800 µL of Reconstitution Buffer, so the powder is now resuspended in a total of 1,650 µL buffer. Pipet up and down to mix. Incubate on ice for 30 minutes. Pipet mix again before use. **Reconstituted Cell Isolation Enzyme Mix should be stored at -20°C and is stable for 1 year after reconstitution**. Thaw reconstituted cell isolation enzyme mix on ice as it is temperature sensitive.
- If working with multiple samples, you may choose **step 11 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

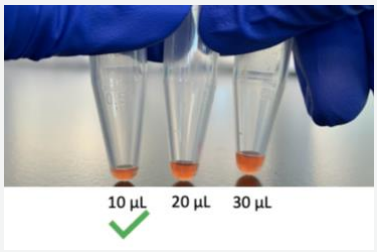
Follow the steps below for Sample Preparation:

1. Harvest the cells, wash with 1X PBS and count.
2. Aliquot 1 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. Aliquot 2.5 µL of the sample into a new tube labeled QC and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

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

## Stage 1B: Sample Preparation - Fresh frozen 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 2: 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

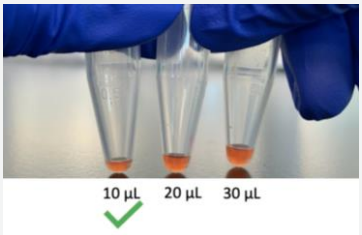
- You may choose to freeze your ground/disrupted tissue pellet at -80°C at this stage. Otherwise, proceed with the protocol.
- All crosslinking reactions (steps 5 – 11) should be carried out at room temperature.

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 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 27 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**




- 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 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.

NOTE **If working with insects,** filter your sample through a 70 µm 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 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.
- **If working with mammalian tissues, refer to Appendix 2 before starting step 12**.

12. Resuspend the pellet in 100 µL 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. 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 |
| **Total** | **52.5 µL** |

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 6 µL 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, or tail) filter your sample through the 70 µm MiniStrainer to remove the cell aggregates. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell/ tissue mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter and discarded. You will be proceeding with the rest of the protocol using the filtrate which should be in single-cell suspension in the tube. If you are not working with muscular tissues, skip this step and proceed to step 22.
22. Aliquot 2.5 µL of the sample into a new tube labeled QC tube and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

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

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

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

- Sample preparation takes ~ 1 hour and 30 minutes.

Before You Begin

- The 10X Wash Buffer and Fragmentation Stop might have precipitated in storage. **Incubate these solutions at 50°C for 15 minutes** or until the precipitate is no longer visible. Vortex to mix prior to use.
- **Dilute the 10X Wash Buffer to 1X** with UltraPure™ Water. Store at room temperature. 1X Wash Buffer is stable at room temperature for 2 months. You need ~15 mL of 1X Wash Buffer per sample for the entire protocol.

  >> Pre-warm 1X Wash Buffer to 37°C for 15 minutes.
- **Prepare 0.3 M DSG in DMSO** (anhydrous) by dissolving 1 mg of DSG in 10.22 µL DMSO. DSG is water-insoluble and moisture-sensitive. Prepare immediately before use. Do not store DSG in solution.
- **Prewarm the Nuclei Prep Buffer at 62°C for 10 minutes before use**. Make sure there is no precipitate visible. Vortex to mix prior to use.
- **Reconstitute Cell Isolation Enzyme Mix** as follows: on ice, transfer 850 µL of Reconstitution Buffer to the tube containing Cell Isolation Enzyme Mix powder. Pipet up and down to mix. Transfer an additional 800 µL of Reconstitution Buffer, so the powder is now resuspended in a total of 1,650 µL buffer. Pipet up and down to mix. Incubate on ice for 30 minutes. Pipet mix again before use. **Reconstituted Cell Isolation Enzyme Mix should be stored at -20°C and is stable for 1 year after reconstitution**. Thaw reconstituted cell isolation enzyme mix on ice as it is temperature sensitive.
- If working with multiple samples, you may choose **step 15 as a SAFE HOLD** step to enable you to proceed with processing the samples in parallel for the remaining steps.

Follow the steps below for Sample Preparation:

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

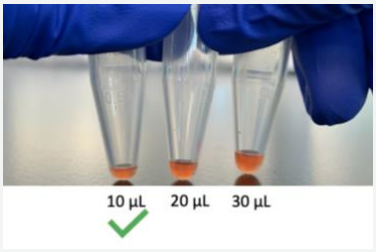
NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be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

6. Take an aliquot to count the cells. Keep the remaining cells on ice until the cells are counted. **Count quickly, this is a delicate sample.**
7. Pipette mix the PBMCs on ice. Transfer a volume equivalent to 1 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. 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. Aliquot 2.5 µL of the sample into a new tube labeled QC and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

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

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

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

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

### Before You Begin

- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood ≤ 24 hours post collection using SepMate™ isolation tubes.
  - SepMate™-15 is designed to process 0.5 to 5 mL of blood samples. Please follow manufacturer's guidelines for volume recommendation.
  - Warm Lymphoprep™ to room temperature (15 - 25°C) before use.
  - Typically, 0.5 to 3 x $10^6$ 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.

   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 µL of 1X Wash Buffer, pipet up and down gently to break up clumps and resuspend the pellet.

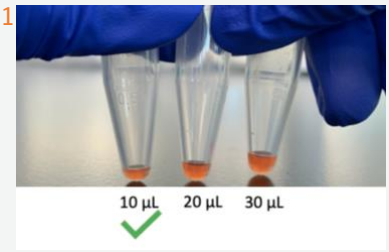
> NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should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

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

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

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

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

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

### Before You Begin

- This protocol is for isolation of Peripheral Blood Mononuclear Cells (PBMCs) from mammalian whole blood 24-72 hours post collection using 10X RBC Lysis Buffer.
  - >> The eBioscience™ 10X RBC Lysis Buffer contains ammonium chloride which is optimal for lysis of erythrocytes with minimal effect on lymphocytes. This preparation method tends to contain higher amounts of cellular debris and may 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 3 mL of eBioscience™ 10X RBC lysis buffer to room temperature 27 mL of molecular grade water. Mix by inverting.
2. Mix the blood sample gently by pipetting. Transfer 3 mL of blood to the 50 mL centrifuge tube containing 30 mL of 1X RBC Lysis Buffer. Mix by inverting.
3. Incubate for 10-15 minutes at room temperature (no more than 15 minutes).
4. Spin the cells at 500 x g for 5 minutes. Discard the supernatant.
5. Resuspend the pellet in 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and resuspend the pellet.

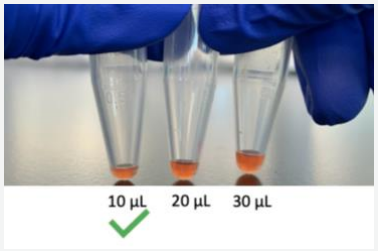
NOTE **If cell clumping is observed at this stage, filter the sample through the MiniStrainer.** Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the cell mixture into the MiniStrainer. Quick spin at 500 x g for 5 seconds. The cell aggregates should be retained in the filter. The PBMCs should be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

6. Centrifuge at 500 x g for 5 minutes. Carefully discard the supernatant.
7. Resuspend the pellet with 200 µL of 1X PBS, pipet up and down gently to break up clumps and resuspend the pellet.
8. Take an aliquot to count the cells. Keep the remaining cells on ice until cells are counted. **Count quickly, this is a delicate sample.**
9. Gently, pipette mix the PBMCs on ice. Transfer 1 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. 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. Aliquot 2.5 µL of the sample into a new tube labeled QC tube and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation ( Stage 2).

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

## Stage 1F: Sample Preparation - Fresh frozen plant leaves

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.
- **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 12 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 30 mg of frozen leaves.
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 – 12) should be carried out at room temperature.

4. Resuspend the tissue pellet in 1 mL of 1X PBS at room temperature and pipet up and down using **wide bore pipet tips** to ensure no clumps are present.
5. Add 10 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 27 µL of 37% formaldehyde.
8. Rotate the tube for 10 minutes at room temperature. Sample should not settle.
9. Spin the tube at 1,000 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.

NOTES

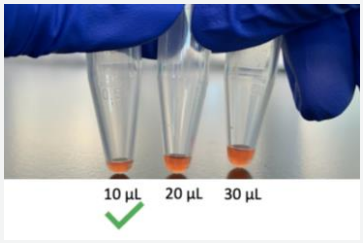
- The centrifugation steps must be carried out in a **swinging bucket rotor**. Using a swinging bucket rotor reduces cell loss and generates the expected QC results.

10. Resuspend the tissue pellet with 700 µL of 1 X Wash Buffer, pipet up and down with **wide bore pipet tips** to break up clumps and fully resuspend the pellet.
11. Filter your sample through a 70 µm MiniStrainer. Place a MiniStrainer in a 1.5 mL microfuge tube. Pipet the sample (resuspended in Wash Buffer) into the MiniStrainer. Quick spin for 5 seconds. Debris should be retained in the filter and discarded. You will proceed to step 12 with the filtrate collected in the tube.
12. Spin the tube at 1,000 *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

- 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




- Step 12 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 13 with all samples in parallel.

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

| Temperature | Time |
|---|---|
| 62°C | 10 minutes |

15. Spin the tube at 1,000 *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.**

16. 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** |

17. 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.

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

| Temperature | Time |
|---|---|
| 65°C | 5 minutes |

20. Quick spin the tube. Add 6 µL of 10% Triton. **Pipet up and down to fully mix.**
21. Incubate the tube on benchtop (at room temperature/no shaking) for 5 minutes. This tube now contains your **LYSATE**.
22. Mix well then aliquot 2.5 µL of the sample into a new tube labeled QC tube and **store at -80°C**. Proceed with the rest of your sample to Proximity Ligation (Stage 2).

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

## Stage 2: Proximity Ligation

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

- Proximity ligation takes ~ 1.5 hour.
- The Crosslink Reversal Buffer might have precipitated in storage. **Incubate at 50°C for 15 minutes** or until the precipitate is no longer visible. Vortex prior to use.
- Follow best practices when working with beads (see Good Practices). The below protocol uses 0.2 mL strip tubes and corresponding magnetic rack. However, the below protocol is compatible with 1.5 mL tubes and corresponding magnetic rack.

Before You Begin

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

### 2.1 Bind Chromatin to Chromatin Capture Beads

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

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Transfer 100 µL of resuspended Chromatin Capture Beads to a **new** 0.2 mL PCR tube.
3. Add your lysate to the Chromatin Capture Beads aliquoted above.
4. Pipet up and down to fully mix. Incubate at room temperature, off the magnetic rack, for 10 minutes.
5. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Discard the supernatant.
6. Remove the tube from the magnetic rack and wash the beads with 150 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.**

### 2.2 Proximity Ligation 1

Follow the steps below for Proximity Ligation 1:

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

| Reagent | Volume Per Reaction |
|---|---|
| UltraPure Water | 40 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 2.1 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
3. Remove the tube from the magnetic rack and **immediately** add to the beads 52.5 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.**

## 2.3 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 2.2 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
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.**

## 2.4 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 2.3 in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant and immediately proceed to the next step (**do not let the beads dry out**).
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.

## 2.5 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<br>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.

## 2.6 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 ≥ 150 ng.
- If the yield you recover is **≥ 150 ng**, proceed to Library Preparation (Stage 3).
- If the yield you recover is **< 150 ng**, you should expect a lower complexity library which may be insufficient to support sequencing up to 300M read pairs. You have the following options:
  1. Continue with the Library Preparation (Stage 3), generate a library and shallow sequence it for QC analysis (see QC Analysis & Sequencing) to assess if the library you generated has sufficient complexity to support sequencing up to 300M read pair depth or if additional library is needed .
  2. Perform the Lysate QC and determine the lysate yield/your sample chromatin content (Refer to Appendix 3: Troubleshooting Guide For Yields).

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

## Stage 3: Library Preparation

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

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

Before You Begin

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

NOTES

- The protocol from here onwards is per **1 LIBRARY** prep. If you are preparing more than 1 library simultaneously, scale the reactions proportional to the number of libraries.
- If you plan to generate multiple libraries (up to 3) from the proximity ligated DNA recovered at the end of Stage 2 to support large genomes (> 3 Gb), split the Stage 2 DNA **evenly** between library preps. We recommend a **minimum input of 150 ng**.
- LinkPrep Library Module **must be used** for library preparation. Other library preparation kits are **NOT** compatible.

### 3.1 Library Prep 1

Follow the steps below for Library Prep 1:

1. Place **up to 1,000 ng of purified DNA** from step 13 Stage 2.6 in a new 0.2 mL PCR tube and bring the volume to 40 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 |
| **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 |

### 3.2 Library Prep 2

Follow the steps below for Library Prep 2:

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

| Reagent | Volume Per Reaction |
|---|---|
| 2X Library Prep 2 Buffer | 50 µL |
| Library Prep 2 Enzyme Mix | 1 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 |

## 3.3 Library Prep 3

Follow the steps below for Library Prep 3:

1. Quick spin the tube. To the tube containing the reaction from library prep 2, **add 2.5 µL of Lib Prep 3 Enzyme Mix to the tube.**

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

| Temperature | Time |
|---|---|
| 37°C | 15 minutes |

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

## 3.4 DNA Purification

Follow the steps below for DNA Purification:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 85 µL of resuspended SPRIselect beads to the 0.2 mL PCR tube containing your sample.
3. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
4. Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
5. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
6. Leave the tube in the magnetic rack and wash the beads **twice** with 200 µL fresh 80% ethanol. Do not resuspend the beads for these washes. Add the ethanol, wait for 1 minute then discard the ethanol supernatant.
7. After the last wash, quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol.
8. Air dry the beads for 3 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
9. Off the magnetic rack, resuspend the beads in 16 µL UltraPure Water.
10. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
11. Incubate at room temperature, off the magnetic rack, for 2 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. **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.

## 3.5 Index PCR

Follow the steps below for Index PCR:

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

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:

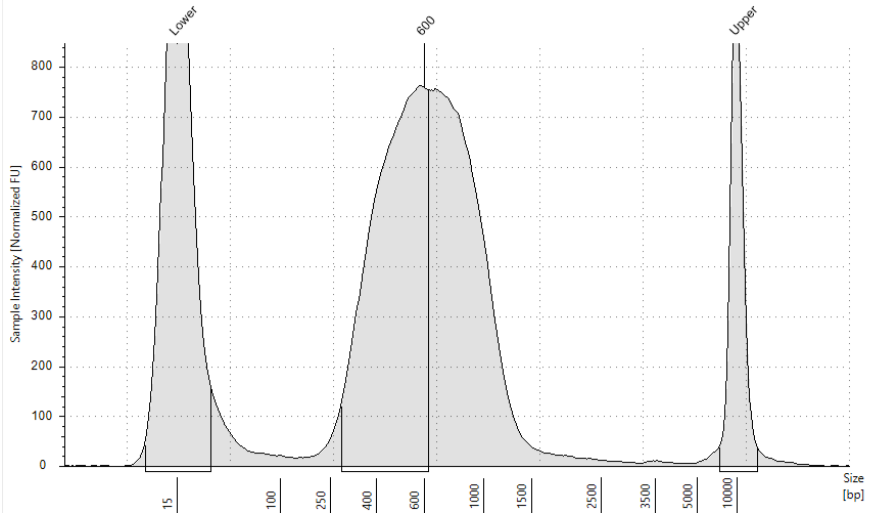
| Step | Temperature | Time | Cycles |
|---|---|---|---|
| Enzyme Activation | 98°C | 3 minutes | 1 |
| Denature | 98°C | 20 seconds | |
| Anneal | 60°C | 30 seconds | 12 |
| Extend | 72°C | 30 seconds | |
| Extend | 72°C | 1 minute | 1 |
| | 12°C | Hold | |

## 3.6 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 > 200 ng if you used >150 ng input into library preparation. If the yield is < 200 ng, refer to the Troubleshooting Guide For Yields (Appendix 3).
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 2).

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




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

## QC Analysis & Sequencing

Dovetail® 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 to determine the long-range information and complexity in the library. While this is recommended, the shallow sequencing for QC analysis is not required. The QC assessment is based on shallow sequenced data (1M - 10M read pairs 2 x 150 bp) generated by a **MiSeq** or **MiniSeq** platform. If you don't have access to a MiSeq or MiniSeq, you can shallow sequence the library on an alternative sequencing platform to assess mappability and long-range information. However, you may not be able to accurately assess the duplication rate. Cantata Bio provides all kit users access to QC analysis pipeline available on readthedocs (https://assemblylink.readthedocs.io/en/latest/).

## Appendix 1: Dual Index Primers

LinkPrep™ Primer Module Set # 1 includes four I5 index primers and six I7 index primers. **Verify that the indexes selected for pooling have the appropriate color balance for your sequencing instrument.**

NOTE LinkPrep Primer Module Set #1 contains sufficient index primers to support multiplexing up to 24 libraries, where each library is prepared by combining a unique I5 Index Primer with a unique I7 Index Primer in steps 2 and 3 of Stage 3.5 Index PCR. For example, 6 libraries can be prepared by setting up 6 individual PCR reactions as follows:

- Library 1: I5 Index Primer 1 and I7 Index Primer 1
- Library 2: I5 Index Primer 1 and I7 Index Primer 2
- Library 3: I5 Index Primer 1 and I7 Index Primer 3
- Library 4: I5 Index Primer 1 and I7 Index Primer 4
- Library 5: I5 Index Primer 1 and I7 Index Primer 5
- Library 6: I5 Index Primer 1 and I7 Index Primer 6

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

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

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

## Appendix 2: Cell Isolation Module

Refer to this appendix when 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 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 in Stage 1B) | 100 µL |
| 100 mM $CaCl_2$ | 5 µL |
| **Total** | **105 µL** |

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

| Temperature | Time |
|---|---|
| 37°C | **30** minutes |

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

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

d. Proceed to Step 12 in Stage 1B: Fresh frozen animal tissue.

## Appendix 3: Troubleshooting Guide For Yields

- This guide enables users to troubleshoot:
  - Purified DNA yield (end of Stage 2) < 150 ng.
  - Library yield (end of Stage 3) < 200 ng.
  - If working with plants, contact support@cantatabio.com to help troubleshoot.

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

**Reason:** Insufficient input material (used less than recommended starting amount or chromatin content in the sample is low).
**Recommendation:** Perform the Lysate QC (Appendix 4) to quantity the chromatin content recovered from the sample and check the fragmentation size distribution.

| Fragmentation profile *within* recommended range and lysate yield < 300 ng | Fragmentation profile *outside* recommended range and lysate yield < 300 ng |
|---|---|
| Redo the sample preparation with **double** the input amount.<br><br>NOTE<br>• You may choose to combine the purified DNA at the end of Stage 2 for library conversion at Stage 3 (refer to Appendix 6 for how to combine samples for library preparation).<br>• You may choose to produce two technical replicate libraries to pool for deep sequencing to 300M read pairs. | Refer to Appendix 5: Troubleshooting Guide For Fragmentation Profile and redo the sample preparation with **double** the input amount.<br>If working with **plants**, contact support@cantatabio.com. |

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

| Reason | Recommendation |
|---|---|
| 1. You started the library preparation with < 150 ng. | 1. Shallow sequence the library and run a QC analysis to assess long-range and complexity. If needed, generate another library to pool for deep sequencing. |
| 2. The chromatin was not properly fragmented. | 2. If the sample was under-fragmented, insufficient proximity ligated molecules are available for library conversion. Perform Lysate QC (Appendix 4) and check the fragment size distribution. Refer to Troubleshooting Guide for under-fragmented samples to improve the fragmentation profile. |
| 3. Low proximity ligation efficiency. | 3. Beside a user error during the preparation, impurities found in some samples can lower the efficiency of the reactions. Filtering the lysate at the end of Stage 1 through a MiniStrainer before proceeding with proximity ligation can help. **If working with plants, contact support@cantatabio.com.** |

## Appendix 4: Lysate QC

- This guide enables users to troubleshoot unexpected yields at Stage 2 or Stage 3 by:
    - Quantifying the lysate yield recovered at the end of sample preparation (Stage 1).
    - Confirming that the chromatin was properly fragmented in Stage 1.
- The QC protocol below is written for the TapeStation; however, it is also compatible with the Bioanalyzer System and Fragment Analyzer. Please refer to the table below for the recommended kits for each system.

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

### Before You Begin

- The Crosslink Reversal Buffer might have precipitated in storage. **Incubate at 50°C for 15 minutes** or until the precipitate is no longer visible. Vortex prior to use.
- Equilibrate the Chromatin Capture Beads to room temperature.

### Follow the steps below for Lysate QC:

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Thaw the QC tube that was stored at -80°C (see Note at the end of Stage 1). Pipet mix the 2.5 µL aliquot.
3. Transfer 50 µL of the resuspended Chromatin Capture Beads to the QC tube containing the 2.5 µL aliquot.
4. Pipet up and down to fully mix. Incubate the QC tube at room temperature, off the magnetic rack, for 10 minutes.
5. Place the tube in the magnetic rack for 5 minutes (or until the solution looks clear). Discard the supernatant.
6. Remove the tube from the magnetic rack and wash the beads with 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads, place the tube in the magnetic rack for 1 minute and discard the supernatant.
7. Repeat step 6 once, for a total of 2 washes.
8. After removing the last wash, add to the QC tube 51.5 µL of a master mix containing the following reagents **added in the order listed**:

| Reagent | Volume Per Reaction |
|---|---|
| Crosslink Reversal Buffer | 50 µL |
| Proteinase K | 1.5 µL |
| **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<br>Note: secure the tube lid to prevent opening during incubation | Hold |

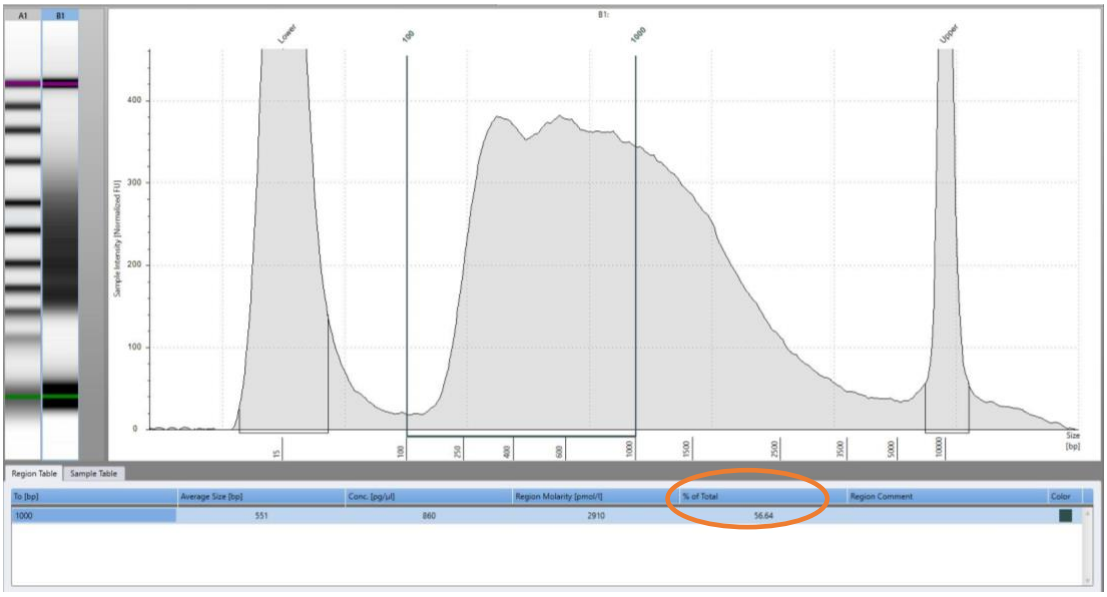
10. Quick spin the tube and place it on the magnetic rack for 1 minute (or until the solution looks clear). **Transfer the SUPERNATANT** to a new 1.5 mL tube for DNA purification. Discard the beads.
11. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
12. Add 90 µL of resuspended SPRIselect 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. We expect a yield > 300 ng. Refer to Appendix 3: Troubleshooting Guide For Yields if the yield you obtain is < 300 ng.
25. Check the fragment size distribution of your purified QC sample on a TapeStation HS D5000 ScreenTape. Make sure your sample is diluted to 1 ng/µL.

- The fragmentation profile should **contain the specified DNA in the range of 100 – 1,000 bp described in the Table below.** On the TapeStation System, create a region from 100 – 1,000 bp (see figure 3 below). Creating this region will automatically generate a '% of total' value. This value should be between 40%-75%. Refer to Appendix 5: Troubleshooting Guide For Fragmentation Profile if the value you obtain is outside of the range recommended for the analysis system you are using.

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% |

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



# Appendix 5: Troubleshooting Guide For Fragmentation Profile

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

## Troubleshooting over-fragmented samples

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

| Protocol | Sample type | Cell Isolation steps to **omit** |
|---|---|---|
| Stage 1A | Cell lines | Stage 1A: steps 12-14 |
| Stage 1B | Fresh frozen mammalian tissues | Stage 1B: Appendix 2 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 µL of 37% formaldehyde instead of 27 µL while keeping all the other steps the same. Ensure **fresh** formaldehyde solution is being used for crosslinking. Formaldehyde solution showing white precipitates should not be used.

## Troubleshooting under-fragmented 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:

1. Perform steps a, b, and c below **before the Nuclei Prep** (i.e before step 12 in Stage 1B) .

   a. Resuspend the tissue pellet in 105 µL of cell isolation master mix containing the following reagents. Pipet up and down to break up clumps and fully resuspend the pellet.

| Reagent | Volume Per Reaction |
|---|---|
| **Reconstituted** Cell Isolation Enzyme Mix (see Before You Begin in Stage 1B) | 100 µL |
| 100 mM $CaCl_2$ | 5 µL |
| **Total** | **105 µL** |

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

| Temperature | Time |
|---|---|
| 37°C | **30** minutes |

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

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

   d. Proceed to Step 12 in Stage 1B: Fresh frozen animal tissue.

2. Filter the sample through the 70 µm 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 6: Combining Samples for Library Preparation

- This guide enables users to:
    - Concentrate their sample if they are combining purified DNA from end of Stage 2 from two proximity ligation reactions for library conversion.

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