Dovetail™ Micro-C Kit

USER GUIDE

VERSION 1.2

DOVETAIL GENOMICS, LLC.
100 ENTERPRISE WAY
SCOTTS VALLEY, CA 95066
(831) 233-3780
SUPPORT@DOVETAIL-GENOMICS.COM

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To learn more about Dovetail's products or services, please contact

info@dovetail-genomics.com

+1 (831) 713-4465

For technical customer support, please contact

support@dovetail-genomics.com

+1 (831) 233-3780

Dovetail™ Micro-C Kit Components and Storage

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

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

Components	Size	Cap Color	Label Color	Storage
TE Buffer pH 8.0	30 mL Bottle			2°C to 8°C
10X Wash Buffer	60 mL Bottle			
TWB Solution	30 mL Bottle			
2X NTB Solution	30 mL Bottle			
LWB Solution	30 mL Bottle			
NWB Solution	30 mL Bottle			
Chromatin Capture Beads	2 mL Tube			
10X Crosslink Reversal Buffer	2 mL Tube			
Streptavidin Beads	0.5 mL Tube			
10X RBC Lysis Buffer	15 mL Tube			
20% SDS	0.5 mL Tube			

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

Components	Tube/Bottle Size	Cap Color	Label Color	Storage
MNase Enzyme Mix	0.5 mL Tube			-30°C to -10°C
10X Nuclease Digest Buffer	2 mL Tube			
100 mM $MgCl_2$	0.5 mL Tube			
0.5 M EGTA	0.5 mL Tube			
End Polishing Enzyme Mix	0.5 mL Tube			
End Polishing Buffer	2 mL Tube			
5X Bridge Ligation Buffer	0.5 mL Tube			
Bridge Ligase	0.5 mL Tube			
Bridge	0.5 mL Tube			
Intra-Aggregate Ligation Enzyme Mix	0.5 mL Tube			
Intra-Aggregate Ligation Buffer	2 mL Tube			
Proteinase K	0.5 mL Tube			
250 mM DTT	0.5 mL Tube			
HotStart PCR Ready Mix	0.5 mL Tube			

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

Optional Add-on Modules: Components and Storage

Dovetail™ Dual Index Primer Set #1 For Illumina®

Table 3. Dovetail™ Dual Index Primer Set #1 For Illumina® (PN DG-PRS-002)

Components	Size	Cap Color	Label Color	Storage
Unique Dual Index (UDI) Primer Pairs (x 8, different)	0.5 mL Tube			-30°C to -10°C

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

Dovetail™ Library Module For Illumina

Table 4. Dovetail™ Library Module For Illumina (PN DG-LIB-001)

Components	Size	Cap Color	Label Color	Storage
End Repair Enzyme Buffer	0.5 mL Tube			-30°C to -10°C
End Repair Enzyme Mix	0.5 mL Tube			
Ligation Enhancer	0.5 mL Tube			
Ligation Enzyme Mix	0.5 mL Tube			
Adaptor for Illumina	0.5 mL Tube			
USER Enzyme Mix	0.5 mL Tube			

* If no color is specified for Label Color or Cap Color, a white or clear label/cap was used.

User Supplied Reagents, Consumables and Equipment

Reagents

Table 5. Reagents

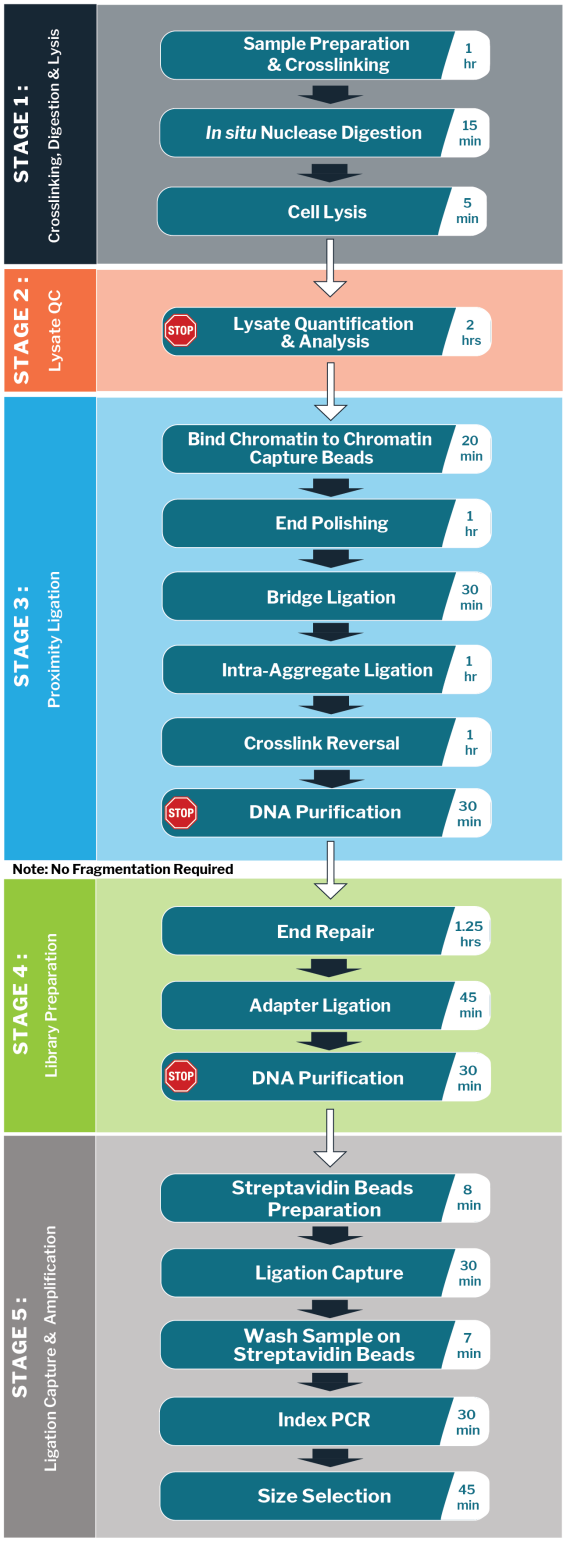
Reagents	Supplier	Part Number
SPRIselect® Beads, 5 mL	Beckman Coulter	B23317
37% Formaldehyde Solution	Sigma-Aldrich	F8775
1X PBS, pH 7.4, 500 mL	Thermo Fisher Scientific	10010023
100% EtOH	Generic	N/A
UltraPure™ DNase / RNase-Free Distilled Water, 500 mL	Thermo Fisher Scientific	10977015
DNA Clean & Concentrator™-5	Zymo Research	D4013
DSG (Disuccinimidyl Glutarate)	Thermo Fisher Scientific	A35392
DMSO (Dimethyl Sulfoxide, Anhydrous ≥ 99.99%)	Sigma-Aldrich	276855-100ML

Consumables and Equipment

Table 6. Consumables and Equipment

Consumables/Equipment	Supplier	Part Number
1.5 mL Low binding microcentrifuge tubes	Generic	
0.2 mL PCR tubes		
5.0 mL centrifuge tubes		
Pipets and pipet tips		
Magnetic separation rack for 0.2 mL and 1.5 mL tubes		
Agitating thermal mixer		
Thermal cycler		
Vortex mixer		
Centrifuge for 0.2 mL, 1.5 mL and 5 mL tubes		
Hemocytometer		
Qubit® Fluorometer	Thermo Fisher Scientific	Q33226
Qubit® dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854
Qubit® Assay Tubes	Thermo Fisher Scientific	Q32856
TapeStation System (Fragment Analyzer or Bioanalyzer)	Agilent	Various

Dovetail™ Micro-C Protocol Overview



Good Practices

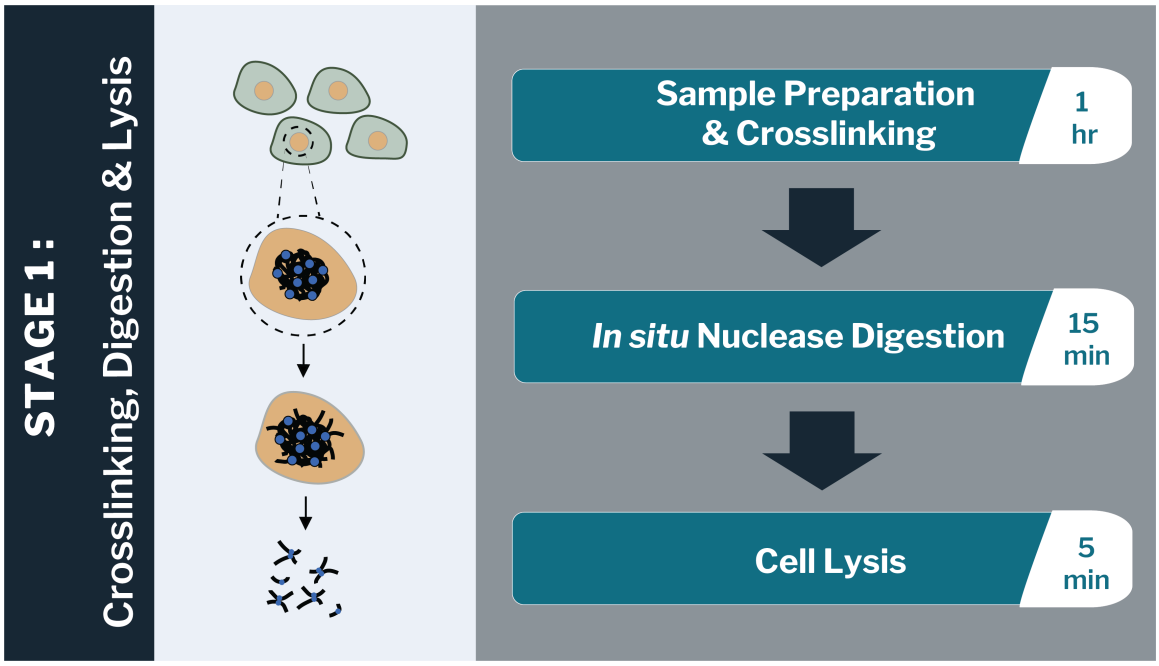
1. Read the entire guide before use, including Before You Begin and the Notes.
2. The cell input amount will influence the efficiency of the digestion reaction. To ensure an accurate cell count, use best practices such as low-speed spins (< 500 x g) using a swinging bucket rotor when harvesting the cells, and counting prior to freezing.
3. To ensure efficient crosslinking, a new or recently opened solution of formaldehyde should be used. Formaldehyde solution containing white precipitates should not be used.
4. Keep all enzymes and master mixes on ice during setup and use. Promptly move reagents back to the indicated storage.
5. Fully thaw buffers, place on ice and thoroughly mix before use.
6. When preparing master mixes, scale the volume of each reagent as appropriate, using 10% excess volume to compensate for pipetting loss.
7. Always add the reagents to the master mix in the specified order as listed throughout the protocol.
8. When working with beads, such as Chromatin Capture, SPRIselect and Streptavidin beads, you should:
    a. Equilibrate the beads to room temperature before use.
    b. Thoroughly vortex the beads immediately before use and ensure they are a homogenous slurry before use.
    c. When placing the tube in the magnetic rack, always wait until the solution looks clear to allow the beads to fully separate before removing the supernatant carefully and slowly. This helps minimize bead/sample loss throughout the protocol.
    d. Do not let the beads dry out during washing steps. Keep the beads in buffer until ready to resuspend them for the next step.
    e. After washing the SPRIselect beads with 80% ethanol during DNA purification, do not let the beads over-dry before proceeding with elution. Over-drying the beads may result in lower recovery of DNA.

Stage 1: Crosslinking, Digestion and Lysis

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

- Sample preparation takes ~ 1.5 hours.

Figure 1. Stage 1: Crosslinking, Digestion & Lysis



Before You Begin

- The 10X Wash Buffer and ● 20% SDS might have precipitated in storage. Incubate these solutions at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.
- Dilute 10X Wash Buffer to 1X with UltraPure™ Water. Store at room temperature. 1X Wash Buffer is stable at room temperature for 2 months. You need ~ 2 mL of 1X Wash Buffer per sample for the entire protocol.
- Prepare 0.3 M DSG in DMSO (anhydrous) by dissolving 1 mg of DSG in 10.22 µL DMSO. DSG is water-insoluble and moisture-sensitive. Prepare **immediately** before use. Do not store DSG in solution.

- Prepare fresh 1X Nuclease Digest Buffer and store at room temperature. 1X Nuclease Digest Buffer is stable for 1 day at room temperature. You need 50 µL of 1X Nuclease Digest Buffer per sample. To prepare 1X Nuclease Digest Buffer (50 µL), mix the following components:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	40 µL	44 µL	x	8	=	352 µL
● 10X Nuclease Digest Buffer	5 µL	5.5 µL	x	8	=	44 µL
● 100 mM $MgCl_2$	5 µL	5.5 µL	x	8	=	44 µL
Total	**50 µL**					

- Set the thermal mixer at 22°C, shaking at 1,250 rpm.
- Thaw ● 0.5 M EGTA at room temperature. Vortex to mix prior to use.

Follow the steps below for Crosslinking, Digestion and Lysis:

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 3,000 x g for 5 minutes. Carefully remove and discard the supernatant.
4. Freeze the cell pellet by placing it at -80°C for at least 30 minutes.

NOTES

- Pre-freezing the cells is required to get an optimal digestion profile.
- All crosslinking reactions (steps 5 – 12) should be carried out at room temperature.

5. Thaw your cell pellet at room temperature then resuspend the pellet in:
   - 1 mL 1X PBS
   - 10 µL 0.3 M DSG
6. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
7. Add 27 µL 37% formaldehyde.
8. Rotate the tube for 10 minutes at room temperature. Cells should not settle.
9. Spin the tube at 3,000 x g for 5 minutes. Carefully remove and discard the supernatant. Use caution, the pellet might be loose.
10. Wash the pellet with 200 µL of 1X Wash Buffer, pipet up and down to break up clumps and fully resuspend the pellet.
11. Spin the tube at 3,000 x g for 5 minutes. Carefully remove and discard the supernatant.
12. Repeat steps 10 and 11 once, for a total of 2 washes.
13. After removing the second wash, resuspend the cell pellet in 50 µL 1X Nuclease Digest Buffer (freshly prepared, see Before You Begin, page 11).
14. Add 0.5 µL of ● MNase Enzyme Mix. Pipet up and down to fully mix.

15. Incubate the tube at 22°C for **exactly** 15 minutes in an agitating thermal mixer set at 1,250 rpm. If you are working with a large number of samples, stagger the start of the digestion for each sample by 20 seconds then stop after corresponding 15 minutes.
16. Stop the reaction by adding 5 µL of ● 0.5 M EGTA. Pipet up and down to fully mix.
17. Add 3 µL of ● 20% SDS to lyse the cells. Pipet up and down to fully mix.
18. Incubate at 22°C for 5 minutes in an agitating thermal mixer set at 1,250 rpm.
19. Continue to Stage 2: Lysate QC, page 13.

Stage 2: Lysate QC

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

- The Lysate QC stage takes ~ 2 hours.
- This stage has 2 objectives:
  - Quantify the lysate to determine the volume of lysate to use in Stage 3.
  - Confirm that the chromatin was properly digested.
- The protocol below is written for the TapeStation; however, it is also compatible with the Bioanalyzer System and Fragment Analyzer. Please refer to the table below for our recommended kits for each system.

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

Figure 2. Stage 2: Lysate QC

Before You Begin

- Verify before use that 100% ethanol was added to the DNA Wash Buffer supplied in the Zymo Research DNA Clean & Concentrator™-5 Kit, as directed by the manufacturer.
- Program the thermal mixer as follows:

Temperature	Time
55°C	15 minutes
68°C	45 minutes
25°C	Hold

» ● 10X Crosslink Reversal Buffer might have precipitated in storage. Incubate at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.

Follow the steps below for Lysate QC:

1. Transfer 2.5 µL of the lysate to a new 1.5 mL tube labeled QC.

   NOTE Store the remainder of the lysate at -80°C. This is the lysate you will be using in Stage 3.

2. Add to the QC tube 51.5 µL of a master mix containing the following reagents **(in order)**:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	45 µL	49. 5 µL	x	8	=	396 µL
● 10X Crosslink Reversal Buffer	5 µL	5.5 µL	x	8	=	44 µL
● Proteinase K	1.5 µL	1.65 µL	x	8	=	13.2 µL
Total	**51.5 µL**					

3. Pipet up and down to fully mix. Incubate the QC tube in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
55°C	15 minutes
68°C	45 minutes
25°C	Hold

4. Purify the QC sample using Zymo Research DNA Clean and Concentrator™-5 Kit (DCC™). Quick spin your QC tube, add 200 µL of DCC™ DNA Binding Buffer, and mix thoroughly.
5. Transfer the mixture to the Zymo-Spin™ Column placed in a collection tube.
6. Centrifuge for 30 seconds at 13,000 x g. Discard the flow-through.
7. Add to the column 200 µL DCC™ DNA Wash Buffer (see Before You Begin, page 13).
8. Centrifuge for 1 minute at 13,000 x g. Discard the flow-through.
9. Repeat steps 7 and 8 once, for a total of 2 washes.
10. Transfer the column to a new 1.5 mL tube.
11. Add 10 µL DCC™ DNA Elution Buffer directly to the column and incubate for 1 minute at room temperature.
12. Centrifuge for 1 minute at 13,000 x g. Discard the column. Your 1.5 mL tube now contains your purified QC DNA.
13. Quantify the purified QC DNA with 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 23.4 (dilution factor)

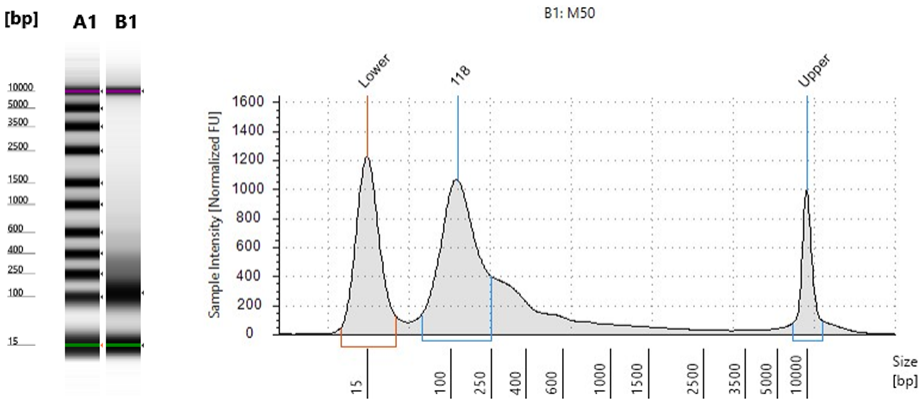
- You will use in Stage 3 a volume of the lysate that corresponds to 1,000 ng. This volume can be calculated as follows:

  $$\text{Volume (µL)} = \frac{1000\ (\text{ng}) \times 58.5\ (\text{µL})}{\text{Total Lysate (ng)}}$$

- If the total lysate amount is < 1,000 ng, use all of the lysate in Stage 3.

14. Check the fragment size distribution of your purified QC sample on a TapeStation D5000 HS ScreenTape. **Make sure your sample is diluted to 1 ng/µL.**
    - For optimal nucleosome-level resolution, the digestion profile should contain 40% - 70% mononucleosomes: the first DNA peak, typically in the size range of 50 - 250 bp for TapeStation, should account for 40% - 70% of total DNA (Figure 3). The size range of the peak may vary for other analytical instruments such as Bioanalyzer and Fragment Analyzer. If the digestion profile contains 40% - 70% mononucleosomes, proceed to Stage 3: Proximity Ligation, page 16.
    - If the digestion profile contains < 40% mononucleosomes, do not proceed with the rest of the protocol. In this case, re-start the protocol and use 2 µL of ● MNase Enzyme Mix instead of 0.5 µL in step 14 in Stage 1: Crosslinking, Digestion and Lysis, page 11.
    - If the digestion profile contains > 70%, you may proceed to Stage 3: Proximity Ligation, page 16 with caution. The library may include a reduced proportion of long-range information. This profile is likely due to suboptimal cell counting or a significant cell loss in the washing steps after crosslinking (steps 9 to 12 in Stage 1: Crosslinking, Digestion and Lysis, page 11).

Figure 3. Expected (QC Pass, 62% mononucleosomes) digestion profile, as analyzed on HS D5000 ScreenTape

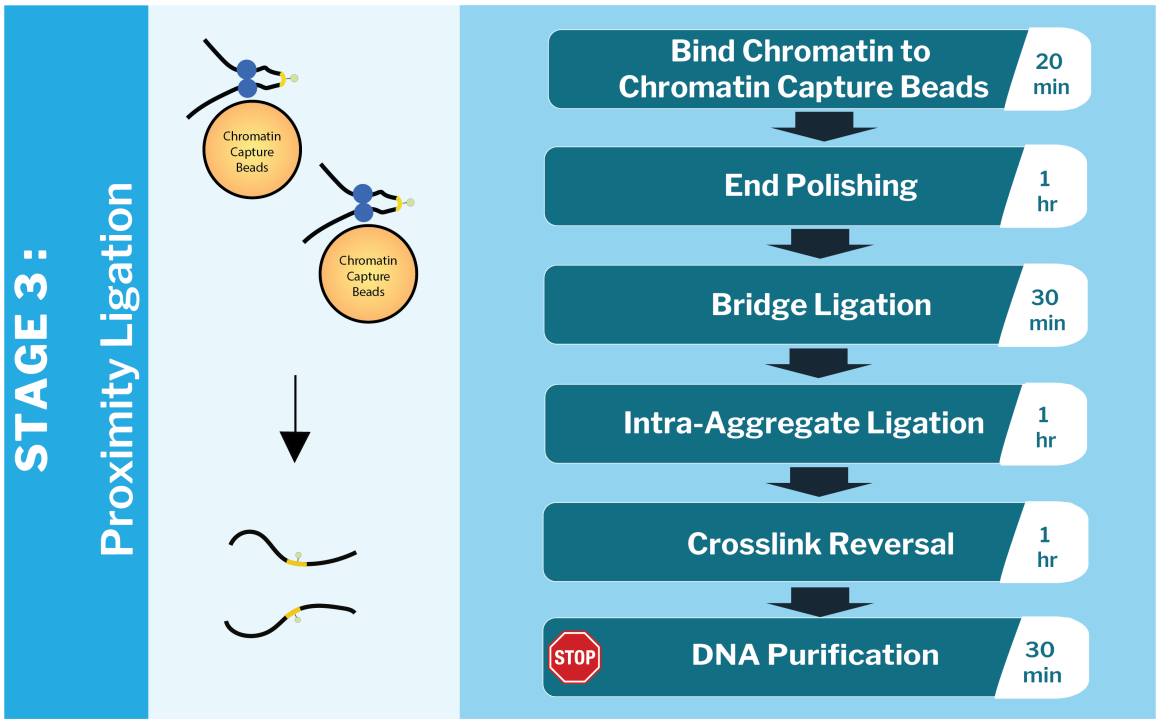


Stage 3: Proximity Ligation

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

- Proximity ligation takes ~ 4.5 hours.
- Follow best practices when working with beads (see Good Practices, page 9).

Figure 4. Stage 3: Proximity Ligation



Before You Begin

- 10X Crosslink Reversal Buffer might have precipitated in storage. Incubate at 37°C for 15 minutes or until the precipitate is no longer visible. Vortex to mix prior to use.
- Thaw End Polishing Buffer, 5X Bridge Ligation Buffer, Bridge, and Intra-Aggregate Ligation Buffer at room temperature. Vortex to mix prior to use.
- Prepare fresh 80% ethanol for DNA purification with SPRIselect beads for optimal results. Fresh preparations of 80% ethanol will also be used in Stage 4, DNA Purification, page 24 and Stage 5, Size Selection, page 27. You need a minimum of 1 mL for all these stages.
- Equilibrate TE Buffer pH 8.0 and Chromatin Capture Beads to room temperature.

3.1 Bind Chromatin to Chromatin Capture Beads

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

1. Equilibrate the Chromatin Capture Beads to room temperature and vortex thoroughly (>30 seconds) to resuspend.
2. Transfer 100 µL of resuspended Chromatin Capture Beads to a new 1.5 mL tube.
3. Add 1,000 ng of the lysate that was stored at -80°C (step 1 NOTE in Stage 2: Lysate QC, page 14) to the 1.5 mL tube containing the beads. If the total lysate amount is <1,000 ng, add all of the lysate.
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. Repeat step 6 once, for a total of 2 washes.

3.2 End Polishing

Follow the steps below for End Polishing:

1. Remove the tube from the magnetic rack and add to the beads 53.5 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
● End Polishing Buffer	50 µL	55 µL	x	8	=	440 µL
● End Polishing Enzyme Mix	3.5 µL	3.85 µL	x	8	=	30.8 µL
Total	**53.5 µL**					

2. Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
22°C	30 minutes
65°C	30 minutes

3. Allow the tube to reach room temperature then place it in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
4. Remove the tube from the magnetic rack and wash the beads once with 150 µL 1X Wash Buffer. Pipet up and down to resuspend the beads, place the tube in the magnetic rack. **Do not remove and discard the supernatant at this step**. Keep the tube in the magnetic rack and the beads in buffer to ensure they do not dry out while you prepare for the next reaction.

3.3 Bridge Ligation

Follow the steps below for Bridge Ligation:

1. Prepare and place on ice **fresh** 50 µL Bridge Ligation Mix by mixing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	35 µL	38.5 µL	x	8	=	308 µL
● 5X Bridge Ligation Buffer	10 µL	11 µL	x	8	=	88 µL
● Bridge	5 µL	5.5 µL	x	8	=	44 µL
Total	**50 µL**					

2. Remove and discard the supernatant from step 4 in 3.2 End Polishing, page 17. Remove the tube from the magnetic rack and add to the beads:

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

3. Pipet up and down to fully mix. Incubate at 22°C for 30 minutes in an agitating thermal mixer set at 1,250 rpm.
4. Place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.
5. Remove the tube from the magnetic rack and wash the beads once 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.

3.4 Intra-Aggregate Ligation

Follow the steps below for Intra-Aggregate Ligation:

1. Remove the tube from the magnetic rack and add to the beads 52 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
● Intra-Aggregate Ligation Buffer	50 µL	55 µL	x	8	=	440 µL
● Intra-Aggregate Ligation Enzyme Mix	2 µL	2.2 µL	x	8	=	17.6 µL
Total	**52 µL**					

2. Pipet up and down to fully mix. Incubate at 22°C for 1 hour in an agitating thermal mixer set at 1,250 rpm.

> **SAFE STOP** For convenience, this ligation reaction can proceed overnight at 22°C in an agitating thermal mixer set at 1,250 rpm.

3. Place the tube in the magnetic rack for 1 minute (or until the solution looks clear). Discard the supernatant.

3.5 Crosslink Reversal

Follow the steps below for Crosslink Reversal:

1. Remove the tube from the magnetic rack and add to the beads 51.5 µL of a master mix containing the following reagents **(in order)**:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	45 µL	49.5 µL	x	8	=	396 µL
● 10X Crosslink Reversal Buffer	5 µL	5.5 µL	x	8	=	44 µL
● Proteinase K	1.5 µL	1.65 µL	x	8	=	13.2 µL
Total	**51.5 µL**					

2. Pipet up and down to fully mix. Incubate in an agitating thermal mixer set at 1,250 rpm as follows:

Temperature	Time
55°C	15 minutes
68°C	45 minutes
25°C	Hold

> **SAFE STOP** For convenience, you can hold at 25°C overnight in an agitating thermal mixer set at 1,250 rpm.

3. Place the tube in the magnetic rack for 1 minute. Transfer 50 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.

3.6 DNA Purification

Follow the steps below for DNA Purification on SPRIselect Beads:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 90 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.

3. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
4. Incubate the tube at room temperature, off the magnetic rack, for 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. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
7. After the last wash, quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol
8. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
9. Off the magnetic rack, resuspend the beads in 52 µL TE Buffer pH 8.0.
10. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
11. Incubate at room temperature, off the magnetic rack, for 5 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. Transfer 50 µL of the **SUPERNATANT** (purified DNA) to a new tube. Discard the beads.
14. Quantify the purified DNA using a Qubit Fluorometer and Qubit dsDNA HS Kit. You should recover a minimum of 50 ng to proceed to Stage 4: Library Preparation, page 21.
15. You will use 150 ng of your purified DNA for Stage 4 in a 50 µL volume. If needed, you can bring up the volume to 50 µL using TE Buffer pH 8.0.
    - If you recovered <150 ng, use all of the purified DNA to proceed to Stage 4.
    - If you recovered >150 ng, use 150 ng to proceed to Stage 4 and keep the remaining purified DNA stored at -20°C. You can use the remaining DNA to prepare additional libraries if your application requires more complexity or coverage.

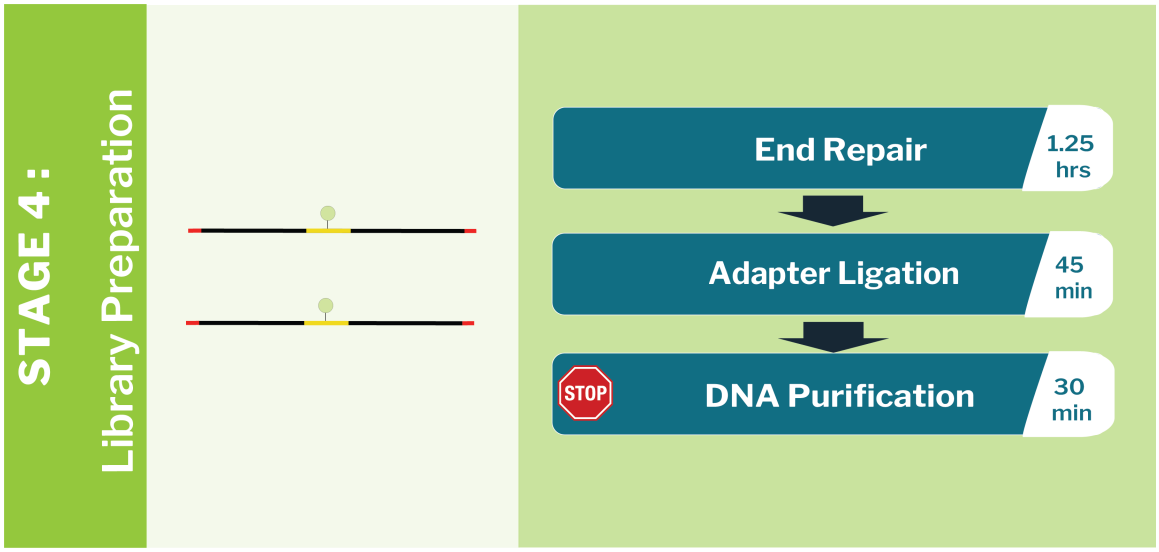
SAFE STOP Purified DNA sample can be stored at -20°C for up to 6 months.

Stage 4: Library Preparation

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

- The library preparation protocol does not require fragmentation.
- The library preparation protocol takes ~ 2.5 hours.
- Follow best practices when working with beads (see Good Practices, page 9).

Figure 5. Stage 4: Library Preparation



Before You Begin

- The ● End Repair Buffer may have precipitated in storage. Incubate for at least 10 minutes at 37˚C until there is no visible precipitate.
- Equilibrate TE Buffer pH 8.0 to room temperature.
- Thaw ● 250 mM DTT and ● Adaptor for Illumina at room temperature. Vortex to mix prior to use.

4.1 End Repair

Follow the steps below for End Repair:

1. Place 50 µL of purified DNA (150 ng) in a 0.2 mL PCR tube.
2. Add to the PCR tube 10.5 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
End Repair Buffer	7 µL	7.7 µL	x	8	=	61.6 µL
End Repair Enzyme Mix	3 µL	3.3 µL	x	8	=	26.4 µL
250 mM DTT	0.5 µL	0.55 µL	x	8	=	4.4 µL
Total	**10.5 µL**					

3. Pipet up and down to fully mix. Quick spin the tube.
4. Incubate in a thermal cycler as follows:

Temperature	Time
20°C	30 minutes
65°C	30 minutes
12°C	Hold

4.2 Adapter Ligation and USER Digest

Follow the steps below for Adapter Ligation and USER Digest:

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

Reagent	Volume Per Reaction
● Adaptor for Illumina	2.5 µL
● Ligation Enzyme Mix	30 µL
● Ligation Enhancer	1 µL
Total	**33.5 µL**

> **NOTE** The Ligation Enzyme Mix and Ligation Enhancer can be mixed ahead of time. The master mix is stable for 8 hours at 4˚C. We do not recommend adding the Adaptor for Illumina to the master mix.

2. Pipet up and down to fully mix. Quick spin the tube.
3. Incubate at 20°C for 15 minutes in a thermal cycler. Hold at 12°C.
4. Following incubation, add 3 µL of ● USER Enzyme Mix to the PCR tube.
5. Pipet up and down to fully mix. Quick spin the tube.
6. Incubate at 37˚C for 15 minutes in a thermal cycler. Hold at 12˚C.

4.3 DNA Purification

Follow the steps below for DNA Purification:

1. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
2. Add 80 µL of resuspended SPRIselect beads to the PCR tube containing the adaptor-ligated sample.
3. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
4. Incubate the tube at room temperature, off the magnetic rack, for 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. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
7. Quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol
8. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
9. Off the magnetic rack, resuspend the beads in 100 µL TE Buffer pH 8.0.
10. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
11. Incubate at room temperature, off the magnetic rack, for 5 minutes.
12. Quick spin the tube and place it in the magnetic rack for 1 minute.
13. Transfer 95 µL of the **SUPERNATANT** (purified adaptor-ligated DNA) to a new tube. Discard the beads.

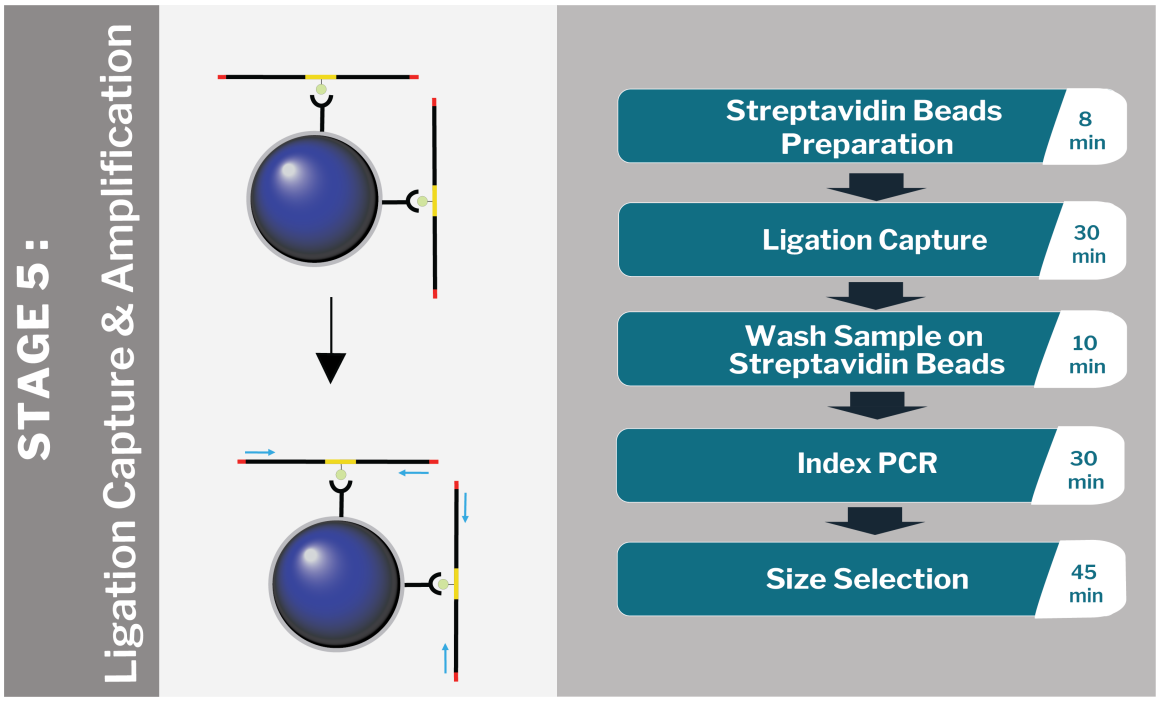
SAFE STOP Purified DNA sample can be stored at -20°C overnight.

Stage 5: Ligation Capture and Amplification

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

- The Ligation Capture and Amplification protocol takes ~ 2 hours.
- Follow best practices when working with beads (see Good Practices, page 9).

Figure 6. Stage 5: Ligation Capture and Amplification



Before You Begin

- Thaw ● UDI Primer Pair and HotStart PCR Ready Mix and **keep on ice while in use.** Vortex to mix prior to use.
- Equilibrate TE Buffer pH 8.0, ● Streptavidin Beads, ■ TWB, ■ 2X NTB, ■ LWB, and ■ NWB to room temperature.

5.1 Streptavidin Beads Preparation

NOTE This step does not involve any DNA sample.

Follow the steps below for Ligation Capture and Amplification:

1. Vortex the ● Streptavidin Beads vial thoroughly (> 30 seconds) to resuspend the beads. Transfer 25 µL of resuspended ● Streptavidin beads to a new 1.5 mL tube.
2. Place the 1.5 mL tube containing the beads in the magnetic rack for 5 minutes. Discard the supernatant.
3. Remove the tube from the magnetic rack and wash the beads with 200 µL ▬ TWB: pipet up and down to resuspend the beads and place the tube in the magnetic rack for 1 minute. Discard the supernatant.
4. Repeat step 3 once, for a total of 2 washes.
5. After the second wash, resuspend the beads in 100 µL ▬ 2X NTB. Pipet up and down to fully mix.

5.2 Ligation Capture

Follow the steps below for Ligation Capture:

1. Transfer the 95 µL of purified adaptor-ligated DNA (from step 13 in 4.3 DNA Purification, page 24) to the 1.5 mL tube containing the Streptavidin beads resuspended in 100 µL of 2X NTB.
2. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
3. Incubate at 25˚C for 30 minutes in an agitating thermal mixer set at 1,250 rpm.

5.3 Wash Sample on Streptavidin Beads

> **NOTE** For each of the washes below, remove the tube from the magnetic rack, add the indicated buffer to the beads, pipet up and down to resuspend the beads, place the tube in the magnetic rack for 1 minute, and discard the supernatant. Remove all of the supernatant between each wash; residual supernatant can interfere with the downstream PCR.

Follow the steps below for Wash Sample on Streptavidin Beads:

1. Quick spin the tube and place it in the magnetic rack for 1 minute. Discard the supernatant.
2. Wash the beads once with 200 µL ▬ LWB.
3. Wash the beads **twice** with 200 µL ▬ NWB.
4. Wash the beads **twice** with 200 µL 1X Wash Buffer.

5.4 Index PCR

> **NOTE** Not all PCR enzymes and master mixes are compatible for amplification in the presence of Streptavidin beads. Please use the HotStart PCR Ready Mix supplied in your Dovetail Kit (Box 2).

Follow the steps below for Index PCR:

1. After the last wash, remove the tube from the magnetic rack and add to the beads 45 µL of a master mix containing the following reagents:

Reagent	Volume Per Reaction	10% Extra		# Reactions		Final
UltraPure Water	20 µL	22 µL	x	8	=	176 µL
HotStart PCR Ready Mix	25 µL	27.5 µL	x	8	=	220 µL
Total	**45 µL**					

2. Add 5 µL ● UDI Primer Pair to the PCR reaction. Use one UDI Primer Pair per PCR reaction (see **Appendix 1: Dual Index Primers, page 29**).
3. Pipet up and down to fully mix then transfer to a new 0.2 mL PCR tube.
4. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle). Place the tube into the thermal cycler and run the following program:

Step	Temperature	Time	Cycles
Enzyme Activation	98°C	3 minutes	1
Denature	98°C	20 seconds	
Anneal	65°C	30 seconds	12
Extend	72°C	30 seconds	
Extend	72°C	1 minute	1
	12°C	Hold	1

5.5 Size Selection

Follow the steps below for Size Selection:

1. Quick spin the PCR tube and place it in the magnetic rack for 1 minute.
2. Transfer 47 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.
3. Add 53 µL of TE Buffer pH 8.0 to the 1.5 mL tube to bring the volume of the sample in the tube to 100 µL.
4. Vortex the SPRIselect beads thoroughly (>30 seconds) to resuspend.
5. Add 50 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.

6. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
7. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
8. Quick spin the tube and place it in the magnetic rack for 5 minutes.
9. Transfer 145 µL of the **SUPERNATANT** to a new 1.5 mL tube. Discard the beads.
10. Add 30 µL of resuspended SPRIselect beads to the 1.5 mL tube containing your sample.
11. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
12. Incubate the tube at room temperature, off the magnetic rack, for 10 minutes.
13. Quick spin the tube and place it in the magnetic rack for 5 minutes. Discard the supernatant.
14. Leave the tube in the magnetic rack and wash the beads **twice** with 200 µL **fresh** 80% ethanol. Do not resuspend the beads for these washes. Simply add the ethanol, wait for 1 minute then discard the ethanol supernatant.
15. Quick spin the tube and place it in the magnetic rack for 1 minute. Use a 10 µL pipet tip to remove traces of ethanol.
16. Air dry the beads for 5 minutes in the magnetic rack until no residual ethanol remains. **Do not over dry the beads**.
17. Off the magnetic rack, resuspend the beads in 30 µL TE Buffer pH 8.0.
18. Vortex to mix thoroughly. Quick spin the tube (make sure to spin down only briefly and to stop before the beads start to settle).
19. Incubate the tube at room temperature, off the magnetic rack, for 5 minutes.
20. Quick spin the tube and place it in the magnetic rack for 1 minute.
21. Transfer 28 µL of the **SUPERNATANT** to a new 1.5 mL tube. The supernatant is your size selected library. Discard the beads.
22. Quantify your size selected library using a Qubit Fluorometer and Qubit dsDNA HS Kit.
23. Use a TapeStation or Bioanalyzer to verify the size distribution of your size selected library. The size range is expected to be between 350 bp and 1,000 bp.

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

Stage 6: Sequencing & QC Analysis of Dovetail™ Micro-C Libraries

Dovetail™ Micro-C libraries are sequenced via Illumina® se**quencers in paired-end mode. Each Micro-C library can be deep sequenced up to 300 M read pairs. We recommend to shallow sequence the library to run a QC analysis prior to deep sequencing.** The QC analysis requires 1 to 2 million (**2 x 75 bp, 2 x 100 bp, or 2 x 150 bp**) read pairs. Dovetail Genomics® provides all kit users with access to QC analysis pipeline available on readthedocs (**https://micro-c.readthedocs.io/en/latest/index.html**).

Appendix 1: Dual Index Primers

Dovetail™ Dual Index Primer Set #1 for Illumina® includes the following eight **UDI Primer Pairs:**

Table 7. UDI Primer Pairs

Primer Name	HiSeq® 2000/2500, MiSeq®, NovaSeq® systems i5 index	HiSeq® 3000, 4000, X, NextSeq®, MiniSeq®, iSeq® systems i5 index	All systems i7 index
UDI Primer Pair 1	ATATGCGC	GCGCATAT	CTGATCGT
UDI Primer Pair 2	TGGTACAG	CTGTACCA	ACTCTCGA
UDI Primer Pair 3	AACCGTTC	GAACGGTT	TGAGCTAG
UDI Primer Pair 4	TAACCGGT	ACCGGTTA	GAGACGAT
UDI Primer Pair 5	GAACATCG	CGATGTTC	CTTGTCGA
UDI Primer Pair 6	CCTTGTAG	CTACAAGG	TTCCAAGG
UDI Primer Pair 7	TCAGGCTT	AAGCCTGA	CGCATGAT
UDI Primer Pair 8	GTTCTCGT	ACGAGAAC	ACGGAACA

The indexes on the UDI Primer Pairs are color-balanced in sets of four (1-4, 5-8). Indexes within each group of four are fully color balanced and can be pooled for sequencing. You can multiplex less than four samples, but verify color balance before pooling.