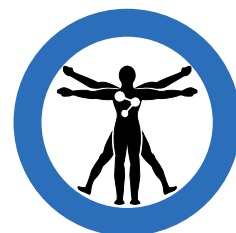




Proximo™ Hi-C Kit

Proximo Hi-C (Human) Kit Protocol



For crude-sample proximity ligation library prep from Human samples, for Illumina® sequencing.

This document applies to Proximo Hi-C (Human) Kit KT4045.

Please review this protocol thoroughly before you start processing your samples. If you have any questions, please contact us at support@phasegenomics.com or visit our [FAQs](#).

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Introduction

Proximity ligation or Hi-C is one of a number of “chromosome conformation capture” (3C) methods, originally designed to study the spatial organization of chromatin.^{1,2} Hi-C employs cost-effective, high-throughput, short-read sequencing to identify the nucleotide sequences of genomic loci that are in close proximity in three-dimensional space, but may be megabases apart in the linear genome sequence. This powerful methodology has enabled significant improvements in genome assembly of humans and other species, as well as structural variant and epigenetic analysis.³ In addition, it has unlocked many applications in metagenomics and microbiology⁴

Phase Genomics' Proximo Platform employs Hi-C to measure the physical proximity between DNA sequences in the same cell. This Proximo Hi-C kit is designed for the preparation of four dual-indexed Hi-C libraries from whole-cell Human samples. The entire protocol, from sample to sequencing-ready library for Illumina paired-end sequencing can be completed in 1.5 to 3 days.

This kit is suitable for all types of whole-cell Human inputs (200K - 2M cells). Any Human sample type (from cultured cells to solid tissue) may be used, but extracted DNA is not a suitable input. Please refer to the **Sample Types and Preparation** section to determine if your type of sample requires additional preparation or reagents.

The Proximo Genome Scaffolding computational tool combines Hi-C sequencing data with draft short- or long-read assemblies assign contigs to scaffolds, arranges contigs in linear order, and then orients contigs in such a way as to maximize the likelihood of having generated the observed Hi-C data. Contact us at support@phasegenomics.com to find out how to use Proximo and FALCON-Phase™ to produce high-quality, chromosome-scale, haplotype-resolved “gold” or “platinum” reference genomes.

The Proximo Platform (library preparation and analysis) is illustrated in Figure 1 on the next page.

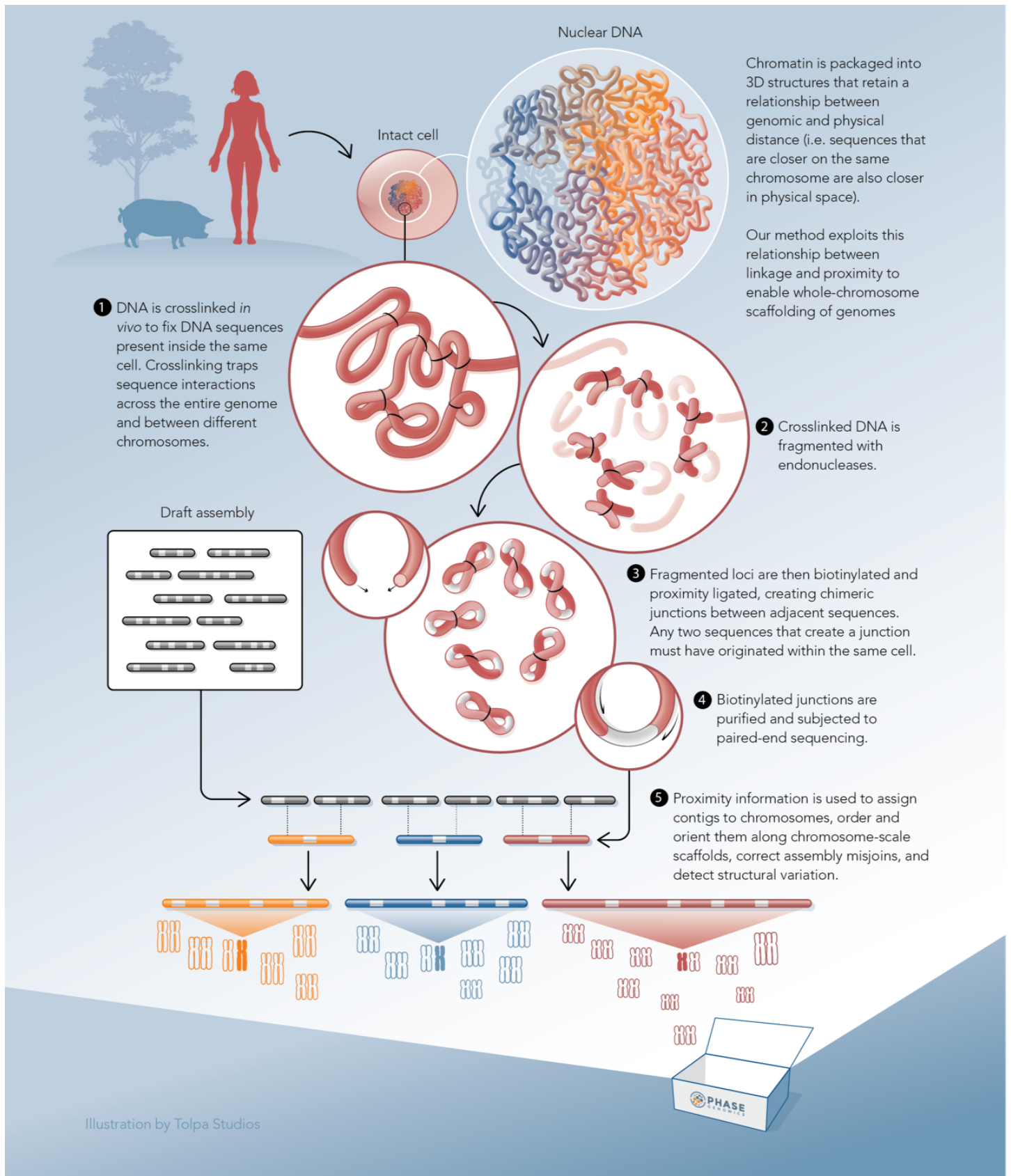


Figure 1. How the Proximo Platform works

References

1. Lieberman-Aiden E, et al. Comprehensive mapping of long-range interactions reveals folding principles of the human genome. *Science* 2009; 326 (5950): 289-293. doi: 10.1126/science.1181369.
2. Van Berkum NL, et al. Hi-C: a method to study the three-dimensional architecture of genomes. *J. Vis. Exp.* 2010; 39: e1869. doi: 10.3791/1869.
3. <http://phasegenomics.com/applications/human-genomics-epigenomics/>
4. <http://phasegenomics.com/applications/metagenomics-microbiology/>

Kit Specifications

Kit Contents

Cap/Label Color	Item Ref #	Cap Label	Tube Label	Volume per tube	No. of Tubes	Storage Temperature (°C)	Used in Step	Before Starting
Red	KS0014	Crosslink Solution	Crosslinking Solution	4 mL	1	-25 to +8 °C	1.2	Thaw and warm to RT
Red	KS0003	Quench Solution	Quenching Solution	1 mL	1	-25 to +25 °C	1.4	Thaw and warm to RT ¹
Orange	KB0036	Lysis Buffer 1	Lysis Buffer 1	1.4 mL	2	-25 to +25 °C	2.1-2.3	Thaw and warm to RT
Orange	KB0003	Lysis Buffer 2	Lysis Buffer 2	1 mL	1	-25 to +25 °C	2.9	Thaw and warm to RT
Yellow	KB0038	Fragment Buffer	Fragmentation Buffer	600 µL	1	-25 to -15 °C	3.3	Thaw on ice
Yellow	KE0039	Fragment Enzyme	Fragmentation Enzyme	10 µL	1	-25 to -15 °C	3.4	Thaw on ice
Yellow	KE0019	Finishing Enzyme	Finishing Enzyme	10 µL	1	-25 to -15 °C	3.6	Thaw on ice
Yellow	KS0010	Stop Solution	Stop Solution	30 µL	1	-25 to -15 °C	3.8	Thaw and warm to RT
Grey	KB0052	10X Ligation Buffer	10X Ligation Buffer	40 µL	1	-25 to -15 °C	4.1	Thaw on ice
Grey	KE0027	Ligation Enzyme	Ligation Enzyme	20 µL	1	-25 to -15 °C	4.2	Thaw on ice
Grey	KE0023	RX Enzyme	RX Enzyme	20 µL	1	-25 to -15 °C	5.1	Thaw on ice
Green	KB0031	Elution Buffer	Elution Buffer	600 µL	1	-25 to -15 °C	6.7, 10, 10	Thaw and warm to RT
Green	KR0012	Recovery Beads	Recovery Beads	1.2 mL	1	+2 to +8 °C	2.11, 6.2, 10.3, 10.6	Warm to RT
Green	KB0046	Recovery Wash Buffer	Recovery Wash Buffer	1 mL	1	-25 to +8 °C	6.4-6.5, 10.8-10.9	Warm to RT. Add 5 mL 95%-100% Ethanol to the provided bottle ²
Blue	KR0010	Strept Beads	Streptavidin Beads	80 µL	1	+2 to +8 °C	7.1	Warm to RT
Blue	KB0029	Bead Bind	Bead Binding Buffer	500 µL	1	-25 to +25 °C	7.4	Thaw and warm to RT
Blue	KB0047	Wash Buffer 1	Wash Buffer 1	4 mL	1	-25 to +25 °C	7.2-7.3, 7.9, 8.15,	Thaw and warm to RT
Blue	KB0048	Wash Buffer 2	Wash Buffer 2	4 mL	1	-25 to +25 °C	7.7-7.8, 8.13-8.14	Thaw and warm to RT
Purple	KB0044	FERAT Buffer	Frag, Repair, A-Tail Buffer	16 µL	1	-25 to -15 °C	8.9	Thaw on ice
Purple	KE0030	FERAT Enzyme	Frag, Repair, A-Tail Enzyme	24 µL	1	-25 to -15 °C	8.6	Thaw on ice
Purple	KE0033	Adapter Ligation	Adapter Ligation Mix	80 µL	1	-25 to -15 °C	8.7	Thaw on ice
Purple	KS0012	Universal Adapter	Universal Adapter	20 µL	1	-25 to -15 °C	8.11	Thaw on ice
Purple	KE0036	Hot Start Mix	Hot Start PCR Mix	100 µL	1	-25 to -15 °C	9.3	Thaw on ice
Purple	KP000N	Primer	PCR Primer Mix	5 µL each	4	-25 to -15 °C	9.2	Thaw on ice
	KB0054	10X CRB	10X CRB	1.6 mL	1	-25 to -15 °C	1.7, 2.8, 2.13, 3.7-3.8, 3.11	Dilute to 1X in molecular biology-grade water before use. ⁴

¹May be warmed to 37 °C to dissolve any precipitate that is present after freezing and thawing, however complete dissolution of precipitate is not necessary for reagent use.

²Prepared Recovery Wash Buffer may be stored at +2 to +8 °C for up to 6 months

³Reference code varies depending on your unique index mixes

⁴1X CRB is stable when stored at room temperature for up to 1 year

Shipping, Storage, and Handling

Proximo Hi-C (Human) Kits are shipped on cold packs. Upon receipt, remove the inner container with the **Recovery Beads** and **Streptavidin Beads**, and store this at +2 to +8 °C. Store the remainder of the kit between -25 and -15 °C. When stored under these conditions, and handled appropriately, all kit components will retain full activity until the expiration date indicated on the kit label.

Always ensure that all components are fully thawed and thoroughly mixed prior to use. Keep all enzymes and Adapter Ligation Mix on ice at all times during use.

Safety Information

When working with chemicals, always wear personal protective gear, such as a lab coat, disposable gloves, and safety glasses. For more information, consult the appropriate safety data sheets (SDS). These are available online at <https://phasegenomics.com/product-literature/>

Other Reagents, Equipment and Consumables Required

Reagents

The following molecular-biology grade reagents are required to complete this protocol. Ensure that reagents are free of DNA, RNA and nucleases.

- 95%-100% ethanol
- Molecular biology-grade water
- Phosphate-buffered Saline

Equipment and Consumables

The following general laboratory equipment and consumables are needed for this protocol.

- Calibrated 2 – 10 μ L pipette and filtered tips
- Calibrated 10 – 100 μ L pipette and filtered tips
- Calibrated 200 – 1000 μ L pipette and filtered tips
- 1.5 or 2 mL microcentrifuge tubes
- 0.2 mL PCR tubes
- Magnetic tube rack/magnet for 2 mL microcentrifuge tubes or 0.2 mL PCR tubes (depending on tube type used in step 2.7).
- Microcentrifuge capable of $\geq 6,000 \times g$
- Thermocycler
- Vortex mixer
- [Qubit™ Fluorometer](#) and [Qubit dsDNA DNA HS Assay Kit](#) (Thermo Fisher Scientific), or similar fluorometric assay for the quantification of double-stranded DNA
- loose fitting Dounce homogenizer or mortar and pestle (see **Sample Types and Preparation** to determine if needed)

Sample Types and Preparation

This protocol is suitable for a wide range of Human types, from cultured cells, to solid tissue and tumors. Consult the table below for required inputs and modifications to the standard protocol for your sample type.

Do not perform any additional mechanical or motorized lysis. Mechanical or motorized homogenizers overly disrupt the sample and severely reduce yields of the final Hi-C library.

Sample Type	Suggested Input	Suggested Lysis Method for Step 2.2
Fibrous tissue	50 mg - 200 mg	Chill in liquid nitrogen or dry ice and grind to a fine powder.
Soft tissue	50 mg - 200 mg	Option 1 (preferred): Homogenize tissue with a loose fitting Dounce in 700 μ L of Lysis Buffer 1 , then transfer the the lysate to a new microcentrifuge tube. Proceed directly to Step 2.4. Option 2: Chill tissue in liquid nitrogen or dry ice and grind to a fine powder. Proceed directly to Step 2.3.
Cells	200K - 2 million cells	No mechanical lysis needed; proceed directly to Step 2.3
Blood	200 - 300 μ L	No mechanical lysis needed; proceed directly to Step 2.3

Workflow Overview

Protocol Notes and Recommended QC

See table in **Sample Types and Preparation**, or in Step 2 (Cell Lysis) to determine optimal lysis method for your sample.

Do not perform any additional mechanical or motorized lysis. These methods overly disrupt the sample and significantly reduce yields for the final Hi-C library.

The sample should be incubated for at least 1 hr, however extending the incubation time up to 16 hr does not negatively affect library yield or quality.

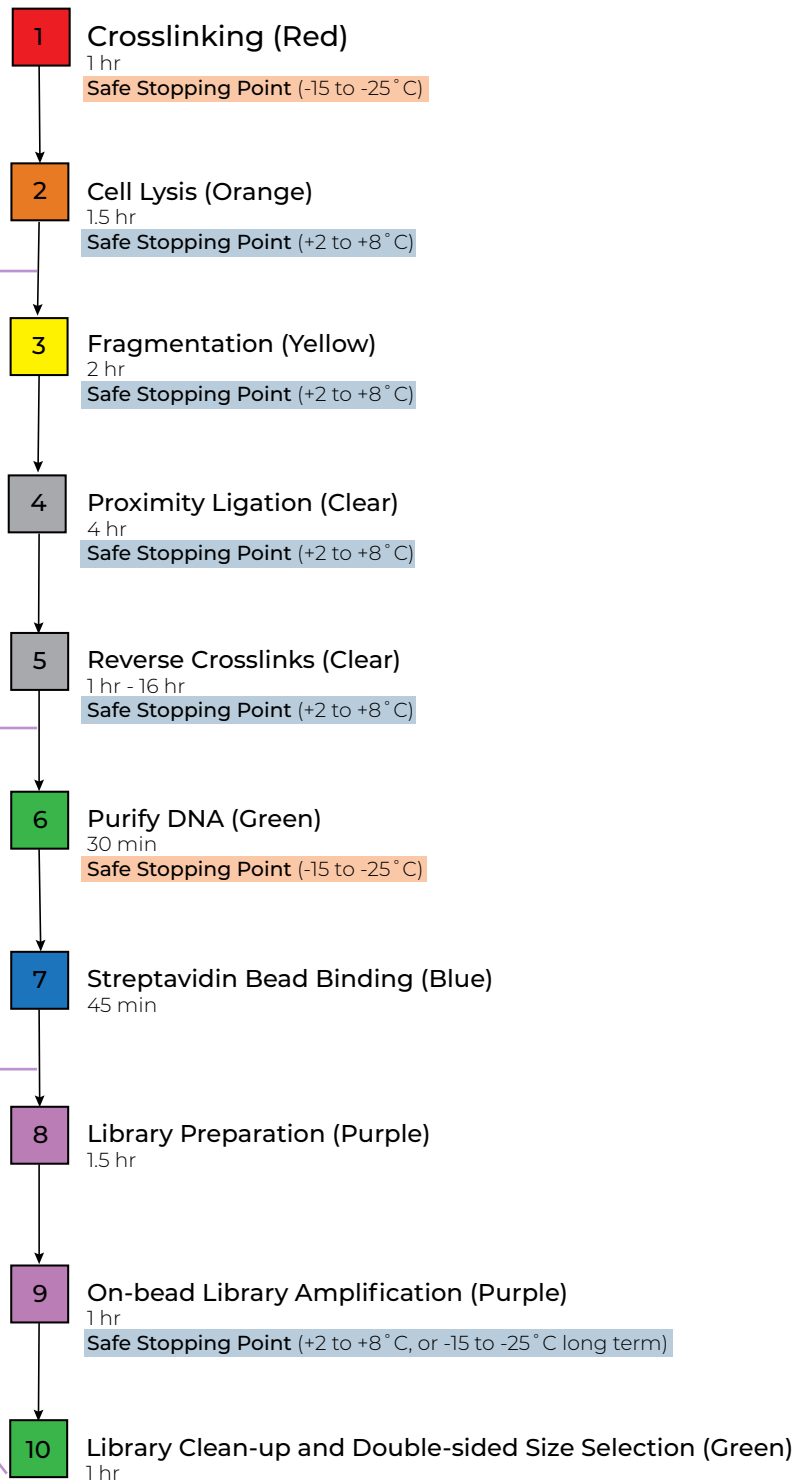
Quantify streptavidin-bound DNA using Qubit dsDNA HS to determine the Universal Adapter dilution and number of PCR cycles.

Quantify library with Qubit dsDNA HS.

Electrophoretic profile of library to assess fragment distribution.

Small amount of sequencing to assess number and quality of Hi-C contacts

Listed times are estimates, including incubations and time at-bench. The listed times assume familiarity with basic molecular biology techniques.



Quick Protocol

This section provides a quick-step guide for experienced users. If this is your first time using the Proximo Hi-C Kit (Human), please refer to the detailed protocol on [p. 17](#).

Step	Protocol	Incubations and notes
1. Crosslinking (Red)	<ul style="list-style-type: none"> Finely chop tissue and transfer tissue to a microcentrifuge tube, then add 1 mL of Crosslinking Solution. For cells or blood, just add solution directly. 	Incubate at room temperature for 15 min while rotating.
	<ul style="list-style-type: none"> Add 100 μL of Quenching Solution. 	Incubate for room temperature for 20 min while rotating.
	<ul style="list-style-type: none"> Centrifuge at 6,000 x g for 5 min to pellet all sample material. Remove and discard the supernatant. Wash the pellet with 1 mL of 1X CRB. Centrifuge at 6,000 x g for 5 min. Carefully remove and discard the supernatant. For tissue: <ul style="list-style-type: none"> Chill in liquid nitrogen or dry ice and grind to a fine powder. o 	
2. Lysis (Orange)	<ul style="list-style-type: none"> Resuspend cells in 700 μL of Lysis Buffer 1. 	Incubate at room temperature for 15 min while rotating.
	<ul style="list-style-type: none"> Centrifuge at 500 x g for 1 min. 	If using cells or blood, this this centrifugation can be skipped.
	<ul style="list-style-type: none"> Transfer the supernatant to a clean microcentrifuge tube. 	The chromatin is in the supernatant.
	<ul style="list-style-type: none"> Centrifuge the supernatant at 6,000 x g for 5 min. Discard the supernatant. 	The chromatin is now in the pellet.
	<ul style="list-style-type: none"> Resuspend the pellet in 500 μL of 1X CRB. Centrifuge at 6,000 x g for 5 min. Carefully remove and discard the supernatant. 	SAFE STOPPING POINT: Pellet may be stored at -25 to -15 °C for up to 1 month.
	<ul style="list-style-type: none"> Resuspend the pellet in 100 μL of Lysis Buffer 2. 	Incubate at 65 °C for 15 min.
	<ul style="list-style-type: none"> Add 100 μL Recovery Beads to sample. 	Incubate at room temperature for 10 min.
	<ul style="list-style-type: none"> Wash the beads: <ul style="list-style-type: none"> Place the sample tube on a magnetic rack Once the solution has cleared, remove and discard the supernatant without disrupting the beads Remove the tube from the magnetic rack and gently resuspend the beads in 200 μL 1X CRB. 	SAFE STOPPING POINT: Store sample at +2 to +8 °C overnight.

Step	Protocol	Incubations and notes
3. Fragmentation (Yellow)	<ul style="list-style-type: none"> Place the sample tube on a magnetic rack Once the solution has cleared, remove the supernatant without disrupting the beads Remove the tube from the magnetic rack and gently resuspend the beads in 148 μL of Fragmentation Buffer. 	
	<ul style="list-style-type: none"> Add 2.5 μL of Fragmentation Enzyme. 	Incubate at 37 °C for 1 hr, then cool to 4 °C
	<ul style="list-style-type: none"> Add 2.5 μL of Finishing Enzyme. 	Incubate at 12 °C for 30 min.
	<ul style="list-style-type: none"> Add 6 μL of Stop Solution. 	
	<ul style="list-style-type: none"> Wash the beads: <ul style="list-style-type: none"> Place the sample tube on a magnetic rack Once the solution has cleared, remove and discard the supernatant without disrupting the beads Remove the tube from the magnetic rack and gently resuspend the beads in 200 μL 1X CRB. Repeat the bead wash steps for a total of 2 washes with 1X CRB. 	SAFE STOPPING POINT: Store bead-bound sample in 1X CRB at +2 to +8 °C overnight.
4. Proximity Ligation (Clear)	<ul style="list-style-type: none"> Remove 1X CRB from beads. Add 85 μL of molecular biology-grade water. Add 10 μL of 10X Ligation Buffer. 	
	<ul style="list-style-type: none"> Add 5 μL of Ligation Enzyme. 	Incubate at 25 °C for 4 hr, followed by 65 °C for 10 min
		SAFE STOPPING POINT: Store sample at +2 to +8 °C overnight.
5. Reverse Crosslinks (Clear)	<ul style="list-style-type: none"> Add 5 μL of RX Enzyme. 	Incubate at 65 °C for 1 - 18 hr
		SAFE STOPPING POINT: Store sample at +2 to +8 °C overnight.

Step	Protocol	Incubations and notes
6. Purify DNA (Green)	<ul style="list-style-type: none"> ■ Add 100 μL of Recovery Beads to the sample tube. 	Incubate at room temp for 10 min.
	<ul style="list-style-type: none"> ■ Rinse the beads: <ul style="list-style-type: none"> ▶ Place the sample tube on a magnetic rack. ▶ Once the solution has cleared, remove and discard the supernatant without disrupting the beads. ▶ Keeping the beads on the magnet, gently rinse the beads with 200 μL of Recovery Wash Buffer without disrupting the beads, leaving the buffer on the beads for 30 sec - 1 min between washes. ■ Repeat the bead wash steps for a total of 2 washes with Recovery Wash Buffer ■ Air dry the beads. 	Leave tubes with caps open on the magnet at room temperature for 10 - 15 min.
	<ul style="list-style-type: none"> ■ Resuspend the beads in 100 μL of Elution Buffer. 	Incubate at room temperature for 5 min.
	<ul style="list-style-type: none"> ■ Place the sample tube on a magnetic tube rack or magnet. ■ Once the solution has cleared, recover the DNA-containing supernatant and transfer to a fresh tube. 	

Step	Protocol	Incubations and notes
7. Streptavidin Bead Binding (Blue)	<p>Prepare the Beads</p> <ul style="list-style-type: none"> ■ Transfer 20 μL of Streptavidin Beads into a new 2 mL microcentrifuge tube (or 0.2 mL PCR tube). ■ Place the tube on a magnetic tube rack or magnet for at least 30 sec. ■ Once the solution has cleared, remove and discard the supernatant without disrupting the beads. ■ Wash the beads: <ul style="list-style-type: none"> ▶ Place the sample tube on a magnetic rack ▶ Once the solution has cleared, remove and discard the supernatant without disrupting the beads ▶ Remove the tube from the magnetic rack and gently resuspend the beads in 200 μL Wash Buffer 1. ■ Repeat the bead wash steps for a total of 2 washes with Wash Buffer 1. ■ Resuspend beads in 100 μL of Bead Binding Buffer. 	
	<p>Bind the Sample to the Beads.</p> <ul style="list-style-type: none"> ■ Transfer 100 μL of purified DNA from step 6 to the washed Streptavidin Beads. 	Incubate at room temperature for 10 min.
	<ul style="list-style-type: none"> ■ Wash the beads: <ul style="list-style-type: none"> ▶ Place the sample tube on a magnetic rack ▶ Once the solution has cleared, remove and discard the supernatant without disrupting the beads ▶ Remove the tube from the magnetic rack and gently resuspend the beads in 200 μL Wash Buffer 2. ■ Repeat the bead wash steps for a total of 2 washes with Wash Buffer 2. ■ Repeat the bead wash steps once with Wash Buffer 1. ■ Resuspend the beads in 200 μL of molecular biology-grade water. ■ Measure the concentration of DNA (while still bound to the streptavidin beads) using a Qubit™ dsDNA HS Assay Kit or similar fluorometric assay. 	

Step	Protocol	Incubations and notes
8. Library Preparation (Purple)	<ul style="list-style-type: none"> ■ Transfer no more than 500 ng of DNA-containing Streptavidin Beads to a fresh microcentrifuge tube. ■ Place the sample tube on a magnetic tube rack or magnet. ■ Once the solution has cleared, remove and discard the supernatant without disrupting the beads. ■ Place tube on pre-cooled thermocycler. 	Pre-cool thermocycler to 4 °C.
	<ul style="list-style-type: none"> ■ To beads add: <ul style="list-style-type: none"> ▶ 40 µL of Molecular biology-grade water ▶ 4 µL of Frag, Repair, & A-Tail Buffer ▶ 6 µL of Frag, Repair, & A-Tail Enzyme 	Fragment, end-repair, and A-tail using thermocycler program listed in Step 8.8 .
	<ul style="list-style-type: none"> ■ To sample add: <ul style="list-style-type: none"> ▶ 5 µL of Universal Adapter (diluted if necessary) ▶ 20 µL Adapter Ligation Mix 	Dilute Universal Adapter according to the table listed in Step 8.9 .
	<ul style="list-style-type: none"> ■ Wash the beads: <ul style="list-style-type: none"> ▶ Place the sample tube on a magnetic rack ▶ Once the solution has cleared, remove and discard the supernatant without disrupting the beads ▶ Remove the tube from the magnetic rack and gently resuspend the beads in 200 µL Wash Buffer 2. ■ Repeat the bead wash steps for a total of 2 washes with Wash Buffer 2. ■ Repeat the bead wash steps once with Wash Buffer 1. ■ Repeat the bead wash steps once with molecular biology-grade water for a total of 7 washes. 	Incubate at 20 °C for 15 min, no heated lid.
9. On-bead Amplification (Purple)	To beads add: <ul style="list-style-type: none"> ■ 20 µL of molecular biology-grade water ■ 25 µL Hot Start PCR Mix ■ 5 µL of one PCR Primer Mix 	Amplify with PCR protocol given in Step 9.4 of the detailed protocol.

Step	Protocol	Incubations and notes
10. Library Clean-up (Clean-up)	<ul style="list-style-type: none"> ■ Place the sample tube on a magnetic tube rack or magnet and allow solution to clear. ■ Transfer 50 µL of the library-containing supernatant to a new tube. 	
	<ul style="list-style-type: none"> ■ Add 55 µL of Recovery Beads. 	Incubate at room temperature for 10 min.
	<ul style="list-style-type: none"> ■ Place the sample tube on a magnetic tube rack or magnet. 	Your library is in the supernatant. Do not discard.
	<ul style="list-style-type: none"> ■ Transfer the supernatant (105 µL) to a new tube containing 17.5 µL of Recovery Beads. 	Incubate at room temperature for 10 min.
	<ul style="list-style-type: none"> ■ Rinse the beads: <ul style="list-style-type: none"> ▶ Place the sample tube on a magnetic rack. ▶ Once the solution has cleared, remove and discard the supernatant without disrupting the beads. ▶ Keeping the beads on the magnet, gently rinse the beads with 200 µL of Recovery Wash Buffer without disrupting the beads, leaving the buffer on the beads for 30 sec - 1 min between washes. ■ Repeat the bead rinse steps for a total of 2 washes with Recovery Wash Buffer ■ Air dry the beads. 	Leave tubes with caps open on the magnet at room temperature for 10 - 15 min.
	<ul style="list-style-type: none"> ■ Resuspend the beads in 30 µL of Elution Buffer. 	Incubate at room temperature for 5 min.
	<ul style="list-style-type: none"> ■ Place the sample tube on a magnetic tube rack or magnet. ■ Once the solution has cleared, recover the Proximo Hi-C library-containing-supernatant and transfer to a fresh microcentrifuge tube. 	See Step 11 in detailed Protocol for recommended QC to determine if your library is sufficient.

Detailed Protocol

1. Crosslinking (Red)

- 1.1 Finely chop 50 - 200 mg of tissue. (Consult **Sample Types and Preparation** if not using solid tissue)

If working with cell pellets or blood, skip step 1.1 and move directly into Step 1.2

- 1.2 Resuspend sample in 1 mL of **Crosslinking Solution**.
- 1.3 Incubate at room temperature for 15 min with occasional mixing by inversion or rotation.
- 1.4 Add 100 µL of **Quenching Solution**.
- 1.5 Incubate at room temperature for 20 min with occasional mixing by inversion or rotation.
- 1.6 Centrifuge at 6,000 x g for 5 min to pellet all sample material. Remove and discard the supernatant.
- 1.7 Wash the pellet with 1 mL of **1X CRB** (prepared as described on [p. 6](#)) and centrifuge at 6,000 x g for 1 min to gently compact the cellular material. Carefully remove and discard the supernatant.

Centrifuge at 1,000 x g for an additional minute if needed to facilitate the removal of supernatant.

SAFE STOPPING POINT: Pellet can be stored at -25 to -15 °C

2. Cell Lysis (Orange)

Pre-heat a heating block, water bath, or thermocycler to 65 °C (for use in Step 2.8). Bring Recovery Beads to room temperature.

- 2.1 Vortex **Lysis Buffer 1** to resuspend any particulates that may have settled out.
- 2.2 The lysis protocol differs slightly for different sample types. Select the method from the table below that is most appropriate for your sample type.

Sample Type	Lysis Method for Step 2.2
Fibrous tissue	Chill in liquid nitrogen or dry ice and grind to a fine powder.
Soft tissue	Option 1 (preferred): Homogenize tissue with a loose fitting Dounce in 700 µL of Lysis Buffer 1 , then transfer the the lysate to a new microcentrifuge tube. Proceed directly to Step 2.4. Option 2: Chill tissue in liquid nitrogen or dry ice and grind to a fine powder. Proceed directly to Step 2.3.
Cells	No mechanical lysis needed*; proceed directly to Step 2.3.
Blood	No mechanical lysis needed*; proceed directly to Step 2.3.

*Do not perform any additional mechanical or motorized lysis. Mechanical or motorized homogenizers overly disrupt the sample and severely reduce yields for the final Hi-C library.

- 2.3 Resuspend the sample in 700 µL of **Lysis Buffer 1** and mix well by vortexing gently or pipetting thoroughly.
- 2.4 Incubate for 20 min at room temperature with occasional mixing by inversion or rotation.
- 2.5 Centrifuge at 500 x g for 1 min. **The chromatin is in the supernatant.**

If working with cell pellets or blood, it is optional to skip step 2.5 and move directly into Step 2.7

- 2.6 Carefully transfer the chromatin-containing supernatant to a fresh microcentrifuge tube. Avoid disturbing the pellet.

Avoid transferring large amounts of debris, but a small amount of tissue debris is not cause for concern.

- 2.7 Centrifuge the supernatant from Step 2.6 at 6,000 x g for 5 min and discard the supernatant. **The chromatin is now in the pellet.**
- 2.8 Resuspend the pellet in 500 µL of **1X CRB** and centrifuge at 6,000 x g for 5 min. Discard the supernatant.

SAFE STOPPING POINT: Sample pellet may be stored at -25 to -15 °C for up to 1 month.

- 2.9 Resuspend the pellet in 100 µL of **Lysis Buffer 2** and transfer the sample to a 0.2 mL PCR tube.
- 2.10 Incubate at 65 °C for 15 min.
- 2.11 Briefly allow sample tube to cool. Thoroughly resuspend **Recovery Beads** and add 100 µL of beads to sample tube. Mix well by vortexing gently or pipetting thoroughly.

*Chromatin binds irreversibly to **Recovery Beads**. The crosslinked DNA-protein complexes will remain bound to the beads until completion of **Step 5: Reverse Crosslinks**.*

- 2.12 Incubate at room temperature for 10 min.
- 2.13 Wash the beads:

- Place the sample tube in a magnetic rack or on a magnet.
- Once the solution has cleared, remove the supernatant without disrupting the beads.
- Remove the tube from the magnet and gently resuspend the beads in 200 µL of **1X CRB**.

If after several minutes your sample is not clearly adhering to the magnet, briefly centrifuge the sample to collect the bead-bound sample in the bottom of your tube and remove the supernatant, avoiding transfer of any particulate sample. Then resuspend the beads in 100 µL of 1X CRB. Repeat as needed until the beads better adhere to the magnet.

SAFE STOPPING POINT: Bead-bound sample may be stored in **1X CRB** at +2 to +8 °C overnight.

3. Fragmentation (Yellow)

Make sure program is setup before beginning. (see Step 3.3-3.6).

- 3.1 Place the sample tube on a magnetic rack.
- 3.2 Once the solution has cleared, remove the supernatant without disrupting the beads.
- 3.3 Remove the tube from the magnetic rack and gently resuspend the beads in 148 µL of **Fragmentation Buffer**.
- 3.4 Add 2.5 µL of **Fragmentation Enzyme** to the sample and mix by vortexing gently or pipetting thoroughly.
- 3.5 Incubate the sample at 37 °C for 1 hr, then cool to 4 °C for at least 1 min.
- 3.6 Once the sample has cooled to 4 °C, add 2.5 µL of **Finishing Enzyme** to the reaction and mix by vortexing gently or pipetting thoroughly.
- 3.7 Incubate at 12 °C for 30 min.
- 3.8 Add 6 µL **Stop Solution** and mix by vortexing gently or pipetting thoroughly to quench the reaction.

Promptly add Stop Solution after 30 minutes at 12°C. Extended incubation at 12°C can result in a low quality library.

- 3.9 Wash the beads:

- Place the sample tube in a magnetic rack or on a magnet.
- Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
- Remove the tube from the magnet and gently resuspend the beads in 200 µL of **1X CRB**.

- 3.10 Repeat the bead wash steps one more time with 200 µL of **1X CRB** per wash, for a total of two washes.

SAFE STOPPING POINT: Store bead-bound sample in **1X CRB** at +2 to +8 °C overnight.

4. Proximity Ligation (Clear)

- 4.1 Place the sample tube on a magnetic rack.
- 4.2 Once the solution has cleared, remove the supernatant without disrupting the beads.
- 4.3 Remove the tube from the magnetic rack and gently resuspend the beads in 85 µL of **Molecular Biology-grade Water** to the bead-bound sample.
- 4.4 Add 10 µL of **10X Ligation Buffer**.
- 4.5 Add 5 µL of **Ligation Enzyme** and mix by vortexing gently or pipetting thoroughly.
- 4.6 Incubate the sample as follows:

Step	Temperature (°C)	Time
Ligation	25	4 hr
Enzyme inactivation	65	10 min
Final hold	4	Hold

SAFE STOPPING POINT: Store sample at +2 to +8 °C overnight.

5. Reverse Crosslinks (Clear)

Heat thermocycler to 65 °C (for use in Step 5.2).

- 5.1 Add 5 µL of **RX Enzyme** to the ligation reaction and mix well by vortexing or pipetting.
- 5.2 Incubate at 65 °C for at least 1 hr (up to 16 hours).

The sample is no longer bound to the beads and has been released into solution.

SAFE STOPPING POINT: The reaction may be incubated at 65 °C overnight, or stored at +2 to +8 °C overnight after the 1 hr incubation at 65 °C.

6. Purify DNA (Green)

Prepare Recovery Wash Buffer by adding 2.5 mL of 95-100% ethanol to the 500 µL of provided Recovery Wash Buffer bottle and mix well.

- 6.1 Allow sample tube to cool to room temperature.
- 6.2 Thoroughly resuspend the **Recovery Beads** and add 100 µL of **Recovery Beads** to the sample tube and mix thoroughly by vortexing or pipetting.
- 6.3 Incubate at room temperature for 10 min.
- 6.4 Rinse the beads:
 - Place the sample tube in a magnetic rack or on a magnet.
 - Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
 - Keeping the beads on the magnet, gently rinse the beads with 200 µL of **Recovery Wash Buffer** without disrupting the beads, leaving the buffer on the beads for 30 sec to 1 min between washes.
- 6.5 Repeat the bead rinse steps for a total of 2 rinses with **Recovery Wash Buffer**.
- 6.6 Air dry the beads at room temperature for 5 - 15 min on the magnet with the cap open.

*Over-drying is not problematic for **Recovery Beads**. Air dry the beads by leaving the tube on the magnet for 5 - 15 min with the cap open.*
- 6.7 Remove the sample tube from the magnet and thoroughly resuspend the beads in 100 µL of **Elution Buffer**.
- 6.8 Incubate at room temperature for 5 minutes to elute the DNA.
- 6.9 Place the sample tube on a magnetic tube rack or magnet.
- 6.10 Once the solution has cleared, recover the **DNA-containing-supernatant** and transfer to a fresh tube. Discard the beads.

SAFE STOPPING POINT: Purified, proximity-ligated DNA may be stored at -25 to -15° C (indefinitely)

7. Streptavidin Bead Binding (Blue)

A. Prepare the Beads

Do not yet combine the beads with the DNA recovered in Step 6. DNA-binding will occur after beads are prepared in section B.

- 7.1 Thoroughly resuspend the **Streptavidin Beads** and transfer 20 μ L into a new microcentrifuge tube (or 0.2 mL PCR tube).
- 7.2 Wash the Beads:
 - Place the sample tube in a magnetic rack or on a magnet.
 - Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
 - Remove the tube from the magnet and gently resuspend the beads in 200 μ L of **Wash Buffer 1**.
- 7.3 Repeat the bead wash steps one more time with 200 μ L of **Wash Buffer 1** for a total of two washes.
- 7.4 Remove beads from the magnet and resuspend in 100 μ L of **Bead Binding Buffer**.

B. Bind the Sample to the Beads

- 7.5 Transfer 100 μ L of purified DNA (from Step 6) to the washed **Streptavidin Beads** (from Step 7.4) and mix by vortexing gently or pipetting thoroughly.
- 7.6 Incubate at room temperature for 10 min, mixing occasionally by gentle vortexing or inversion.
- 7.7 Wash the beads:
- Place the sample tube in a magnetic rack or on a magnet.
 - Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
 - Remove the tube from the magnet and gently resuspend the beads in 200 μ L of **Wash Buffer 2**.
- 7.8 Repeat the bead wash steps one more time with 200 μ L of **Wash Buffer 2** for a total of two washes.
- 7.9 Repeat the bead wash steps one more time with 200 μ L of **Wash Buffer 1**.
- 7.10 Repeat the bead wash steps one more time with 200 μ L of **molecular biology-grade water**.
- 7.11 With your bead-bound sample suspended in 200 μ L of water, measure the concentration of DNA (while still bound to the streptavidin beads) using a Qubit™ dsDNA HS Assay Kit or similar fluorometric assay.

It is essential that the beads are well resuspended in the molecular biology-grade water prior to quantification by fluorometry. Vortex the beads in the fluorometric assay tube immediately prior to measuring DNA concentration to ensure an accurate measurement.

Beads will interfere with spectrophotometric quantitation of bound DNA. Use of fluorometric assay is a requirement.

A concentration of less than 10 ng at this stage does NOT necessarily indicate failure. Proceed through the remainder of the protocol as written.

8. Library Preparation (Purple)

Pre-cool a thermocycler to 4 °C (see Step 8.8).

- 8.1 Transfer no more than 500 ng of streptavidin-bound DNA to a fresh microcentrifuge tube.
- 8.2 Place the sample tube in a magnetic rack or on a magnet.
- 8.3 Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
- 8.4 Resuspend the beads in 40 µL of **molecular biology-grade water**.
- 8.5 Place the sample in the pre-cooled thermocycler and then cool to 4 °C for at least 1 min.
- 8.6 Add 4 µL of **Frag, Repair, & A-Tail Buffer**.
- 8.7 Add 6 µL of **Frag, Repair, & A-Tail Enzyme** and mix by vortexing gently or pipetting thoroughly.

Vortex for at least 5 sec or pipette at least 25 µL of the reaction up and down a minimum of 10 times to ensure proper mixing.

Thorough mixing at this stage is extremely important! Improper mixing will result in a poorly fragmented library and will negatively affect your sequencable yield.

- 8.8 Proceed to fragmentation, end-repair, and A-tailing according to the following program:

Step	Temperature (°C)	Time (min)
Lid temperature	105	
Pre-cooling	4	Hold
Fragmentation, end-repair, and A-tailing	30	5
	65	30
Final hold	4	Hold

- 8.9 If the amount of library measured at step 7.11 was less than 10 ng, dilute the **Universal Adapter (provided tube is 15 µM)** as according to the table below.

Either molecular biology-grade water or 10 mM Tris-HCl, pH 8.0 can be used for the dilution.

Input Mass (ng)*	Adapter Concentration	Volume Water or Tris (µL)	Volume 15 µM Adapter (µM)
> 10	15 µM	do not dilute	
1 - 10	3 µM	4	1
< 1	0.3 µM	49	1

*Measured in Step 7.11

8.10 Add 5 µL of **Universal Adapter** (see step 8.9 for dilution instructions) to the sample and mix by vortexing gently or pipetting thoroughly.

8.11 Add 20 µL of **Adapter Ligation Mix**. Mix by pipetting thoroughly.

*Do not vortex **Adapter Ligation Mix**.*

8.12 Incubate the sample as follows:

Step	Temperature (°C)	Time (min)
Lid temperature	off	
Ligation	20	15

8.13 Wash the beads:

- Place the sample tube in a magnetic rack or on a magnet.
- Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
- Remove the tube from the magnet and gently resuspend the beads in 200 µL of **Wash Buffer 2**.

8.14 Repeat the bead wash steps one more time with 200 µL of **Wash Buffer 2** for a total of two washes.

8.15 Repeat the bead wash steps one more time with 200 µL of **Wash Buffer 1**.

8.16 Repeat the bead wash steps one more time with 200 µL of **molecular biology-grade water**.

9. On-bead Library Amplification (Purple)

- 9.1 Thoroughly resuspend the beads in 20 μ L of **molecular biology-grade water**.
- 9.2 Add 5 μ L one **PCR Primer Mix** and mix by vortexing gently or pipetting thoroughly.

Use a different primer for each sample. Sufficient primers with unique index sequences are provided with each kit. See [Index Sequences](#) for more information).

- 9.3 Add 25 μ L of **Hot Start PCR Mix**.
- 9.4 Amplify the library in a thermocycler programmed as follows:

Step	Temperature (°C)	Time (sec)	Cycles
Initial denaturation	98	45	1
Denaturation	98	15	12*
Annealing	60	30	
Extension	72	30	
Final extension	72	60	1
Hold	12	hold	

*If less than 10 ng DNA was carried into Step 8 (Library preparation), increase the number of PCR cycles to 14

SAFE STOPPING POINT: PCR reaction can be held overnight at +2 to +8 °C, or stored at -25 to -15 °C (indefinitely)

10. Library Clean-up and Double-sided Size Selection (Green)

Use Recovery Wash Buffer Prepared at Step 6.

- 10.1 Place the sample tube on a magnetic tube rack or magnet.
- 10.2 Once the solution has cleared, transfer the **library-containing supernatant** to a new tube.

*Streptavidin beads can be stored in **1X CRB** for troubleshooting if needed. Otherwise they can be discarded.*

- 10.3 Add 57.5 μL (1.15X volume) of thoroughly resuspended **Recovery Beads** to the tube containing the library (from Step 10.2).

Unwanted high molecular weight fragments will be binding to the beads.

- 10.4 Incubate at room temperature for 10 min.
- 10.5 Place the sample tube on a magnetic tube rack or magnet. **Your library is in the supernatant. Do not discard.**
- 10.6 After 2 min, or once the solution has cleared, transfer the supernatant (105 μL) to a new tube containing 15 μL of **Recovery Beads**.

The library is now binding to the beads, leaving unwanted small fragments in the supernatant..

- 10.7 Incubate at room temperature for 10 min.
- 10.8 Rinse the beads:

- Place the sample tube in a magnetic rack or on a magnet.
- Once the solution has cleared, remove and discard the supernatant without disrupting the beads.
- Keeping the beads on the magnet, gently rinse the beads with 200 μL of **Recovery Wash Buffer** without disrupting the beads, leaving the buffer on the beads for 30 sec - 1 min between washes.

- 10.9 Repeat the bead rinse steps for a total of two rinses with **Recovery Wash Buffer**. Air dry the beads at room temperature 10 - 15 min on the magnet with the cap open.

*Over-drying is not problematic for **Recovery Beads**. Air dry the beads by leaving the tube on the magnet for 5 - 15 min with the cap open.*

- 10.10 Remove the sample tube from the magnet and thoroughly resuspend the beads in 30 μ L of **Elution Buffer**.
- 10.11 Incubate at room temperature for 5 min to elute the DNA.
- 10.12 Place the sample tube on a magnetic tube rack or magnet.
- 10.13 Once the solution has cleared, recover the **Proximo Hi-C Library-containing supernatant** and transfer to a fresh microcentrifuge tube. Discard the beads.

11. Library QC (recommended)

- 11.1 Measure the concentration of DNA using a Qubit™ dsDNA HS Assay Kit or similar fluorometric assay.

Yields over 0.5 ng/μL are a strong indication that library preparation has been successful. The library can be stored at -15 to -25 °C indefinitely.

- 11.2 Assess library fragment size using BioAnalyzer or similar instrument.

Before performing a full sequencing run, it is highly recommended that you perform low-pass sequencing (approximately 1 million read pairs) to assess the quality of your Hi-C library. These data can be analyzed using our open-source Hi-C analysis tools (available from https://github.com/phasegenomics/hic_qc).

12. Sequencing

Proximo Hi-C libraries are compatible with any Illumina® sequencer

Genome Size	Sequencing Recommendation
< 0.4 Gbp	> 100 million pairs (2 x 75 bp or longer)
0.4 Gbp – 1.5 Gbp	> 150 million pairs (2 x 75 bp or longer)
> 1.5 Gbp	> 200 Million pairs (2 x 75 bp or longer)

Note: these are meant as guidelines for the amount of data required to scaffold genomes. The actual requirements will vary between genomes and are dependent on assembly quality.

13. Analysis

Take advantage of our expertise! Interested in additional computational analyses? Contact us to learn more about the services listed below:

Proximo SV

Identify large-scale structural variation and determine epigenetic changes using Hi-C data.

FALCON-Phase™

Integrate PacBio long-read assemblies with Hi-C data to generate phased, diploid genome assemblies and services.

Index Sequences

Your kit contains four sets of indexed primers which are used to generate unique dual-indexed Illumina^(R)-compatible libraries with different sequence combinations. If you plan to pool your Hi-C libraries with other libraries for sequencing, please follow standard guidelines for multiplexed sequencing on your specific Illumina[®] instrument.

Please contact us at support@phasegenomics.com if additional primers or assistance with multiplexed sequencing are needed.

Primer Mix	i7 Equivalent Index	i5 Equivalent Index (For iSeq, NovaSeq, MiSeq, and HiSeq 2000/2500)	i5 Equivalent Index For MiniSeq, NextSeq, and HiSeq 3000/4000)
A1	TCAAGATC	TGACGTAG	CTACGTCA
B1	GAGCGCCA	AACTCTCC	GGAGAGTT
D1	ACGACAGA	AGTCTGGT	ACCAGACT
E1	TAATGATG	GTATCGAA	TTCGATAC
F1	ACATTACC	AGTACAGG	CCTGTACT
G1	CAGTCGAC	ACTAGCCT	AGGCTAGT
H1	TGTCGTTT	TCCTAGCA	TGCTAGGA
A2	CAAAGTGT	CGAGTTGC	GCAACTCG
B2	GCGCGGTG	ACCCGACC	GGTCGGGT
C2	AGTGTGTG	GAAATTTT	AAAATTTT
A3	TCAATCCG	ACTGCGAA	TTCGCAGT
B3	CGCTACAT	TAGTCTCG	CGAGACTA
C3	GATCCACT	TGAGCTGT	ACAGCTCA
D3	ATCCACGA	AGTATGCC	GGCATACT
E3	ACGATCAG	TGGTGAAG	CTTCACCA
F3	GTCCTAAG	TACTGCTC	GAGCAGTA
G3	CAACTCCA	ACTCCTAC	GTAGGAGT
H3	AAGCATCG	TACTCCAG	CTGGAGTA
I3	GAAGACTG	TCACCTAG	CTAGGTGA
J3	GAACGGTT	GATCTTGC	GCAAGATC
K3	CTCTATCG	AAGCCTGA	TCAGGCTT
L3	ATGCCTAG	AGTACACG	CGTGTACT
A4	CCACATTG	CGACACTT	AAGTGTCC
B4	ATGTGGAC	CTCACCAA	TTGGTGAG
C4	TGAGACGA	AACCAGAG	CTCTGGTT

Primer Mix	i7 Equivalent Index	i5 Equivalent Index (For iSeq, NovaSeq, MiSeq, and HiSeq 2000/2500)	i5 Equivalent Index For MiniSeq, NextSeq, and HiSeq 3000/4000)
D4	GGTTGGTA	GCGTATCA	TGATACGC
E4	CATCAACC	AATGACGC	GCGTCATT
F4	GCAATTCC	CCACAACA	TGTTGTGG
G4	ACCTCTTC	GTATTCCG	CGGAATAC
H4	TTCACGGA	AGGTAGGA	TCCTACCT
I4	CTGGTCAT	ACGAGAAC	GTTCTCGT
J4	CCTATTGG	TGACAACC	GGTTGTCA
K4	AAGACCGT	CTTAGGAC	GTCCTAAG
L4	GGTGTACA	CCGCTTAA	TTAAGCGG

Restriction Enzymes

Restriction Enzyme	Cut Sequence
DpnII	GATC
DdeI	CTNAG
MseI	TTAA
HinfI	GANTC

Revision History

Version	Date	Revision Description
2.0.1	2019-11	<ul style="list-style-type: none"> Corrected "SPRI" Spelling Corrected "kit contents" volumes and tube numbers Corrected pre-heat temperatures for "RX Crosslinking"
	2020-01	<ul style="list-style-type: none"> Changed SPRI bead color from white to green Removed index "C1" from index table Corrected spacing error on p 6. Added note about mechanical lysis Added note about warming quenching solution
3.0	2020-02	<ul style="list-style-type: none"> Exchanged reagent "SPRI beads" for "Recovery Beads" and adjusted corresponding volumes Ethanol wash now with diluted Recovery Wash Buffer Fragmentation Enzyme and Buffer reagents were adjusted; both volume and component change Lysis Buffer 1 reagent was reformulated Tabulated multi-step incubations Expanded index list Decreased number of CRB washes after bead clean-up from 3 to 2 Increased first lysis step from 10 min to 20 min Increased recommended spin speed and time during crosslinking from 1k 1 min to 6k 5 min Updated water description from "deionized" to "molecular biology-grade" Corrected storage instructions after lysis to storage of beads at 2-8 °C General formatting update - font changed to match website
4.0	2020-10	<ul style="list-style-type: none"> Library Preparation and On-bead Library Amplification steps protocol re-formulated Decreased Ligation Enzyme volume Updated introduction Updated links and redirects Removed Bead Reagent addition in Streptavidin Bead Binding Decreased sample input requirements Added quick protocol for experienced users Modified Workflow Overview
	2021-02	<ul style="list-style-type: none"> Corrected Kit ID on cover page from KT6040 to KT4040 Added units to Ligation incubation table Decreased input for 16 cycles of PCR from 20 ng to 10 ng Moved note about removing CRB before ligation for clarity Expanded note about precipitate in Quenching solution.

Version	Date	Revision Description
4.5	2022-12	<ul style="list-style-type: none"> • Added restriction enzyme information • Clarified buffer removal before fragmentation • Clarified buffer removal before ligation • Decreased maximum recommended PCR cycle number from 16 to 14 • Clarified dilution of CRB with molecular biology-grade water • Updated formulation for crosslinking solution, 10X CRB, and Fragmentation Enzyme • Updated volumes for Wash Buffer 1 and Wash Buffer 2 • Corrected typo in step 8.9 • Updated color scheme and kit sticker image • Changed “Animal” to “animal” in introduction • Corrected font error in Kit Specifications • Updated formatting in quick protocol • Corrected typo in Equipment and Consumables (end of sentence was incorrectly deleted in previous iteration of the protocol) • Updated notes about DNA addition in step 8 Step 8.9 - dilution information was moved to an in-line note. • 9.2 - updated wording around number of provided primers
	2023-01	<ul style="list-style-type: none"> • corrected language in brief protocol in crosslinking and fragmentation steps for better clarify • Corrected capitalization error in step 2.11 • Corrected language and spelling error in subheader for streptavidin bead preparation • corrected “repar” to “repair” in table in step 8.8 • Corrected capitalization error in step 10.9 • Corrected grammatical error in Falcon-PHASE description • corrected typo in Notices section
4.5.1	2023-07	<ul style="list-style-type: none"> • Corrected numbering error at the beginning of step 4 • Corrected spelling of “botttom” to “bottom” on p19 • Added clarification about low on-bead measurements at step 7 • Updated final Recovery Bead clean up bead-to-volume ratios

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