PHASE GENOMICS

Proximo™ Hi-C Kit

Proximo Hi-C (Animal) Kit Protocol

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

This document can be used with the following versions of the Proximo Hi-C (Animal) Kits:

- KT2045
- KT9045

Please review this protocol thoroughly before you start processing your samples. If you have any questions, please contact us at support@phasegenomics.com.

Version 5.0

August 2026

For research use only. Not for use in diagnostic procedures.

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Table of Contents

Proximo™ Hi-C Kit (Animal) Protocol v5.0

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 animals and other species, as well as structural variant and epigenetic analysis.[3] In addition, it has unlocked many applications in metagenomics and microbiology.[4]

This Proximo Hi-C kit is designed for the preparation of two (KT2045) or eight (KT9045) dual-indexed Hi-C libraries from whole-cell animal 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 animal inputs. Any animal sample type (from blood or solid tissue to whole insects) 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.

See **14. Analysis** for suggestions on how to analyze or process your data, or contact us at support@phasegenomics.com

The Proximo methodology is illustrated in Figure 1 on the next page.

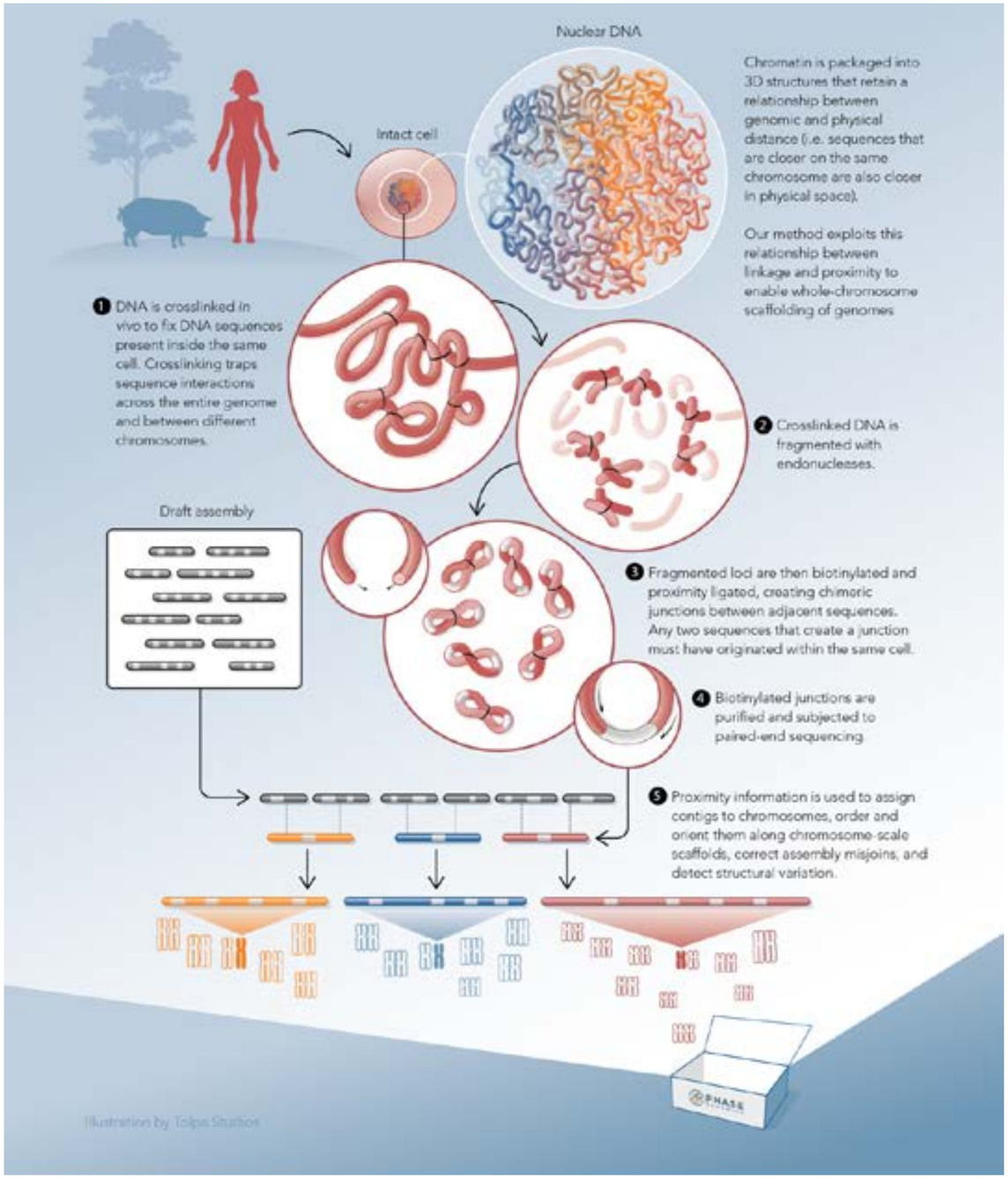


Figure 1. How the Proximo methodology works

Proximo™ Hi-C Kit (Animal) Protocol v5.0

References

1. Lieberman-Aiden E, et al. Comprehensive mapping of long-range interactions reveals folding principles of the Animal 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

Reagent Storage and Preparation

Cap/Label Color	Cap Label	Tube Label	Storage Temperature (˚C)	Used in Step	Before Starting
	PGShield™	PGShield™	-25 to +25˚C	1.3	Thaw and warm to RT
	Crosslink Solution	Crosslinking Solution	-25 to +8˚C	2.3	Thaw and warm to RT
	Quench Solution	Quenching Solution	-25 to +25˚C	2.5	Thaw and warm to RT[1]
	Lysis Buffer 1	Lysis Buffer 1	-25 to +25˚C	3.1-3.2, 3.7	Thaw and warm to RT
	Lysis Buffer 2	Lysis Buffer 2	-25 to +25˚C	3.9	Thaw and warm to RT
	Fragment Buffer	Fragmentation Buffer	-25 to -15˚C	4.1	Thaw on ice
	Fragment Enzyme	Fragmentation Enzyme	-25 to -15˚C	4.1	Thaw on ice
	Finishing Enzyme	Finishing Enzyme	-25 to -15˚C	4.6	Thaw on ice
	Stop Solution	Stop Solution	-25 to -15˚C	4.8	Thaw and warm to RT
	Ligation Buffer	10X Ligation Buffer	-25 to -15˚C	5.1	Thaw on ice
	Ligation Enzyme	Ligation Enzyme	-25 to -15˚C	5.1	Thaw on ice
	RX Enzyme	RX Enzyme	-25 to -15˚C	6.1	Thaw on ice
	Elution Buffer	Elution Buffer	-25 to -15˚C	7.7, 11.10	Thaw and warm to RT
	Recovery Beads	Recovery Beads	+2 to +8˚C	3.11, 7.2, 11.3, 11.6	Warm to RT
	Recovery Wash Buffer	Recovery Wash Buffer	-25 to +8˚C	7.5, 11.8-11.9	Warm to RT. Add 95%-100% Ethanol according to the instructions on the bottle.[2]
	Strept Beads	Streptavidin Beads	+2 to +8˚C	8.1	Warm to RT
	Bead Bind	Bead Binding Buffer	-25 to +25˚C	8.4	Thaw and warm to RT
	Wash Buffer 1	Wash Buffer 1	-25 to +25˚C	8.2-8.3, 8.9, 9.16	Thaw and warm to RT
	Wash Buffer 2	Wash Buffer 2	-25 to +25˚C	8.7-8.8, 9.14-9.15	Thaw and warm to RT
	Universal Adapter	Universal Adapter	-25 to -15˚C	9.10-9.11	Thaw on ice
	FERAT Buffer	Frag, End-repair, A-Tail Buffer	-25 to -15˚C	9.7	Thaw on ice
	FERAT Enzyme	Frag, End-repair, A-Tail Enzyme	-25 to -15˚C	9.7	Thaw on ice
	Adapter Ligation	Adapter Ligation Mix	-25 to -15˚C	9.12	Thaw on ice
	2X PCR Mix	2X PCR Mix	-25 to -15˚C	10.5	Thaw on ice
	Primer	PCR Primer Mix	-25 to -15˚C	10.4	Thaw on ice
	10X CRB	10X CRB	-25 to -15˚C	2.8, 3.8, 3.13, 4.9-4.10	Dilute to 1X in molecular biology-grade water before use.[4]

[1]May 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.
[2]Prepared Recovery Wash Buffer may be stored at +2 to +8˚C for up to 6 months
[3]Reference code varies depending on your unique index mixes
[4]1X CRB is stable when stored at room temperature for up to 1 year

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Kit Contents

		KT2045			KT9045		
-25˚ to -15˚C Box							
Cap/Label Color	Tube Label	Item Ref #	Volume per tube	No. of Tubes	Item Ref #	Volume per tube	No. of Tubes
	Crosslinking Solution	KS0015/KS0024	1 mL/1.5 mL	2	KS0016	10 mL	1
	Quenching Solution	KS0003/KS0026	1 mL/1.8 mL	1	KS0003/KS0026	1 mL/1.8 mL	1
	Lysis Buffer 1	KB0073/KB0014	14 mL/4 mL	1/3	A01024	50 mL	1
	Lysis Buffer 2	KB0003/KB0056	500 µL/1.2 mL	1	KB0003/KB0056	500 µL/1.2 mL	1
	Fragmentation Buffer	KB0006	300 µL	1	KB0068	1.2 mL	1
	Fragmentation Enzyme	KE0038	5 µL	1	KE0047	20 µL	1
	Finishing Enzyme	KE0016	5 µL	1	KE0048	20 µL	1
	Stop Solution	KS0004/KS0020	15 µL/180 µL	1	KS0023/KS0020	50 µL/180 µL	1
	10X Ligation Buffer	KB0051	20 µL	1	KB0053	80 µL	1
	Ligation Enzyme	KE0026	10 µL	1	KE0028	40 µL	1
	RX Enzyme	KE0007	10 µL	1	KE0017	40 µL	1
	Elution Buffer	KB0015/KB0059	350 µL/1.7 mL	1	KB0028/KB0059	1.1 mL/1.7 mL	1
	Bead Binding Buffer	KB0012/KB0061	250 µL/1.5 mL	1	KB0025/KB0061	800 µL/1.5 mL	1
	Wash Buffer 1	KB0047/KB0062	7 mL/10 mL	1	KB0047/KB0062	7 mL/10 mL	1
	Wash Buffer 2	KB0048/KB0063	7 mL/10 mL	1	KB0048/KB0063	7 mL/10 mL	1
	Frag, End-Repair, A-Tail Buffer	KB0043	8 µL	1	KB0045	32 µL	1
	Frag, End-Repair, A-Tail Enzyme	KE0029	12 µL	1	KE0031	48 µL	1
	Universal Adapter	KS0011	10 µL	1	KS0013	40 µL	1
	Adapter Ligation Mix	KE0032	40 µL	1	KE0034	160 µL	1
	2X PCR Mix	KE0035	50 µL	1	KE0037	200 µL	1
	PCR Primer Mix	KP000N	5 µL each	2	KP000N	5 µL each	8
	10X CRB	KB0069	1 mL	1	KB0069	1 mL	1
+2˚ to +8˚C Box							
	Recovery Beads	KR0011	600 µL	1	KR0012	1.2 mL	2
	Recovery Wash Buffer	KB0041/KB0060	500 µL/2 mL	1	KB0060	2 mL	1
	Streptavidin Beads	KR0002	40 µL	1	KR0005	160 µL	1
	PGShield™	KR0013	10 mL	1	KR0013	10 mL	1

Shipping, Storage, and Handling

Proximo Hi-C (Animal) Kits are shipped on cold packs. Upon receipt, remove the container with the **Recovery Beads and Streptavidin Beads**, and store at +2 to +8˚C. If either the **Recovery Beads and Streptavidin Beads** are stored at freezing temperatures (below 0˚C) they will no longer functional and should be replaced. 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/

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Phase Genomics PGShield™

Phase Genomics PGShield™ is a new preservative system for room temperature stabilization of biological samples. Unlike many lysis-based approaches, PGShield™ is designed to keep cells and cell structures intact until processing.

General Properties

- Nontoxic to humans and the environment
- Nonflammable
- Bacteriostatic/Bacteriocidal
- Slightly viscous
- Extended storage conditions
  - -80°C indefinitely
  - +4°C up to 1 year
  - Room temperature for up to 4 months

Sample types

- Cultured cells
- Tissues & cells
- Purified white blood cells
  - Blood will coagulate quickly in PGShield™ making the sample difficult to work with. We recommend removing Red Blood Cells (RBCs) before using PGShield™. We have not tested if heparin stops this effect.

Important Notes for Use

PGShield™ is best used as an immersive agent for cells/tissue at maximum concentration. Do not dilute PGShield™ before use and keep it at no less than 90% concentration.

For blood samples, **lysing red blood cells prior to resuspending in PGShield™ is required**.

For tissue we recommend liquid nitrogen grinding prior to use and complete immersion with enough PGShield™ to fully saturate. While the actual volume required may vary by sample type, we recommend starting with 1 mL PGShield™ to 300 mg tissue.

Additional 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% ethanol
- Molecular biology-grade water
- Liquid nitrogen or dry ice

Equipment and Consumables

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

- Calibrated 2 – 10 µL pipette and filtered tips
- Calibrated 10 – 100 µL pipette and filtered tips
- Calibrated 200 – 1000 µL pipette and filtered tips
- 2 mL microcentrifuge tubes
- 0.2 mL PCR tubes
- Magnetic tube rack/magnet for 0.2 mL PCR tubes and for 2 mL microcentrifuge tubes (if in step 3.13 the bead-bound material is in 2 mL tubes).
- Microcentrifuge capable of ≥10,000 *x 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

Additional Reagents and Equipment

The following molecular biology-grade reagents may be recommended or required for certain sample type inputs. See **Sample Types and Preparation** to determine what additional reagents your library prep may require.

- Red Blood Cell Lysis Buffer
- 1X Phosphate-buffered saline (PBS), pH 7.4
- PGShield™ (included)
- Loose fitting Dounce homogenizer or mortar and pestle

Sample Types and Preparation

This protocol is suitable for a wide range of animal types, from blood or tissue to insects. Consult the table below for required inputs and modifications to the standard protocol for your sample type. Please note that these are guidelines and optimization may still be required for your sample(s).

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	recommended tissue type	Before you start:
Fibrous tissue	50 - 500 mg	• Muscle	• Chill in liquid nitrogen or dry ice and grind to a fine powder. • Proceed to **1. Recommended Pre-Processing** if using PGShield™, otherwise proceed to **2. Crosslinking.**
Soft tissue	50 - 500 mg	• Brain (preferred) • Liver • Kidney	• **If tissue is firm or you do not have access to a loose-fitting dounce**, chill in liquid nitrogen or dry ice and grind to a fine powder • ***If you will be using a dounce:*** roughly chop tissue. Full tissue disruption will be performed at Step 3.2 (See instructions in **3. Cell Lysis**) • Proceed to **1. Recommended Pre-Processing** if using PGShield™, otherwise proceed to **2. Crosslinking.**
Cells	200K - 2 million cells		• Count cells, pellet, and remove media. • Wash once with 1X PBS (not included) • Make sure as much media or buffer is removed as possible before proceeding • Proceed to **1. Recommended Pre-Processing** if using PGShield™, otherwise proceed to **2. Crosslinking.**

[1]For PGShield™ use, begin at Step 1. Recommended Pre-Processing on p. 21.

Sample Type	Suggested Input	recommended tissue type	Before you start:
Blood (nucleated)	200 - 300 µL		• No tissue disruption or mechanical lysis needed. • **Do not put whole blood in PGShield™ .** • Gently pellet cells and rinse pellet once with 1X PBS (not provided). • Proceed with cell pellet of between 200K - 2 million cells. • Proceed to **1. Recommended Pre-Processing** if using PGShield™, otherwise proceed to **2. Crosslinking.**
Blood (enucleated)	200 - 300 µL		• **Do not put whole blood in PGShield™ .** • Proceed with white blood cell pellet of between 200K - 2 million cells. • Perform red blood cell lysis according to manufacturer instructions (Materials not included). • Proceed to **1. Recommended Pre-Processing** (only if RBC lysis was performed) or **2. Crosslinking.**
Insects	50 - 500 mg OR 1-2 large individuals OR 10 - 50 small individuals combined	• Whole organsims • Legs • Body segments • Pupae or larvae recommended	• Chill in liquid nitrogen or dry ice and grind to a fine powder. • Avoid using gut tissue or particularly chitinous tissue if possible. • Then proceed to **2. Crosslinking**.

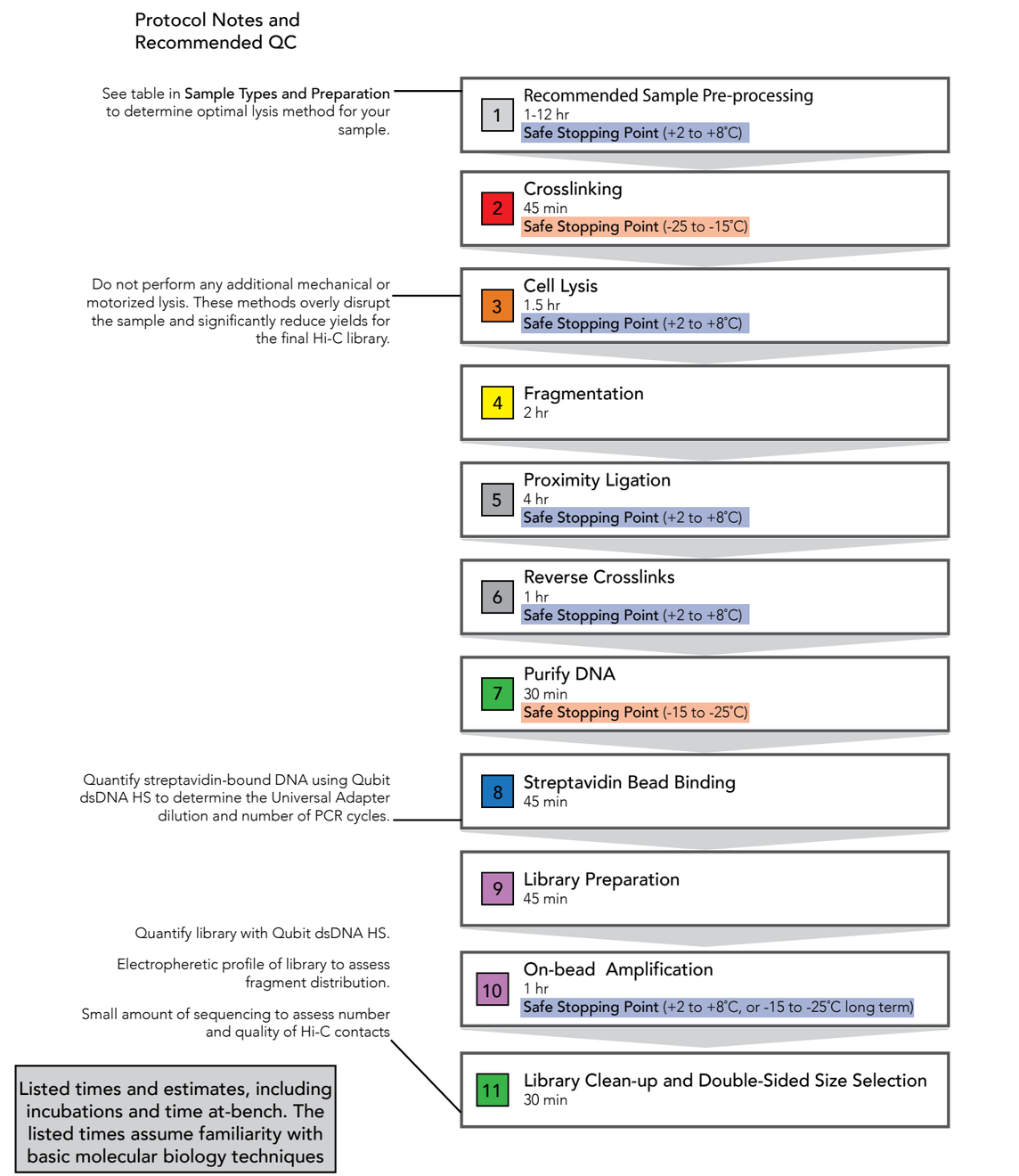
[1]For PGShield™ use, begin at Step 1. Recommended Pre-Processing on p. 21.

Before You Begin

- Determine the best pre-processing for your particular sample
- Determine if you will be using the **Detailed Protocol** instead of the **Quick Protocol** (First-time users should use the **Detailed Protocol**)
- Gather all necessary materials and reagents
- Dilute 10X CRB to 1X CRB with molecular biology-grade water.
- Make sure no reagents were damaged in transit or improperly stored.
- If this is your first time using a Phase Genomics Hi-C Kit, we recommend preparing just one sample with sufficient back-up material in case a re-prep is required
- Pre-program thermocycler programs from steps **4. Fragmentation, 5. Proximity Ligation, 6. RX Crosslinks, 9. Library Preparation**, and **10. On-bead Amplification.**

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Workflow Overview



Quick Protocol

This section provides a quick-step guide for experienced users. If this is your first time using the Proximo Hi-C Kit (Animal), please refer to the detailed protocol on *p. 21**.*

Step	Protocol	Incubations and notes
1. Recommended Sample Pre-processing	■ Liquid nitrogen grind tissue and transfer to a microcentrifuge tube. ■ Add 1 mL **PGShield™** to ground tissue and mix well.	Incubate at 4˚C for a minimum of 1-12 hours
2. Crosslinking (Red)	■ Centrifuge at 10,000 *x g* for 5 min to pellet all sample material. ■ Remove and discard the supernatant.	
	■ Add 1 mL of **Crosslinking Solution**.	Incubate at room temperature for 20 min while rotating.
	■ Add 100 µL of **Quenching Solution**.	Incubate at room temperature for 15 min while rotating.
	■ Centrifuge at 10,000 *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 10,000 *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.

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Step	Protocol	Incubations and notes
3. Lysis (Orange)	■ Resuspend cells in 700 µL of **Lysis Buffer 1.**	Incubate at room temperature for 15 min while rotating.
	■ Centrifuge at 500 *x g* for 30 sec - 1 min.	If using cells, blood, low-input or precious sample, this centrifugation can be skipped.
	■ Transfer the supernatant to a clean microcentrifuge tube.	**The chromatin is in the supernatant.**
	■ Centrifuge the supernatant at 10,000 *x g* for 5 min. ■ Discard the supernatant.	**The chromatin is now in the pellet.**
	■ Optional: Wash the sample pellet with 500 µL of **Lysis Buffer 1** ► Resuspend the pellet in 500 µL of Lysis Buffer 1. ► Centrifuge at 10,000 x g for 5 min ► Discard the supernatant. ► Repeat washes up to 3 times, or until supernatant is clear.	This is recommended for samples with high fat content, adult insects, or any sample that typically performs poorly in standard DNA extractions
	■ Resuspend the pellet in 500 µL of **1X CRB**. ■ Centrifuge at 10,000 *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.
	■ Resuspend the pellet in 100 µL of **Lysis Buffer 2**.	Incubate at 65˚C for 15 min.
	■ Add 100 µL **Recovery Beads** to sample.	Incubate at room temperature for 10 min.
	■ Wash the beads: ► Place the sample 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 150 µL **1X CRB.**	**SAFE STOPPING POINT**: Store sample at +2 to +8˚C overnight in 1X CRB

Step	Protocol	Incubations and notes
4. Fragmentation (Yellow)	▪ Place the sample 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**.	
	▪ Add 2.5 µL of **Fragmentation Enzyme**.	Incubate at 37˚C for 1 hr, then cool to 4˚C
	▪ Add 2.5 µL of **Finishing Enzyme.**	Incubate at 12˚C for 30 min.
	▪ Add 6 µL of **Stop Solution.**	
	▪ Wash the beads: ▪ Place the sample 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 150 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.
5. Proximity Ligation (Clear)	▪ Remove **1X CRB** from beads. ▪ Add 85 µL of **molecular biology-grade water.** ▪ Add 10 µL of **10X Ligation Buffer**.	
	▪ 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.
6. Reverse Crosslinks (Clear)	▪ Add 4 µL of **RX Enzyme.**	Incubate at 65˚C for 1 hr
		SAFE STOPPING POINT: Store sample at +2 to +8˚C overnight.

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Step	Protocol	Incubations and notes
7. Purify DNA (Green)	▪ Add 100 µL of **Recovery Beads** to the sample.	Incubate at room temp for 10 min.
	▪ Rinse the beads: ▸ Place the sample on a magnetic rack. ▸ Once the solution has cleared, remove 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.
	▪ Resuspend the beads in 100 µL of **Elution Buffer**.	Incubate at room temperature for 5 min.
	▪ Place the sample on a magnetic tube rack or magnet. ▪ Once the solution has cleared, recover the **DNA-containing supernatant** and transfer to a fresh tube.	**SAFE STOPPING POINT**: Purified, proximity-ligated DNA may be stored at -25 to -15˚C (indefinitely)

Step	Protocol	Incubations and notes
8. Streptavidin Bead Binding (Blue)	**Prepare the Beads** ■ 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: ▸ Place the sample 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 150 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**.	
	Bind the Sample to the Beads. ■ Transfer 100 µL of purified DNA from step 6 to the washed Streptavidin Beads.	Incubate at room temperature for 10 min.
	■ Wash the beads: ▸ Place the sample 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 150 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.	It is recommended to use 2 µL of bead-bound sample suspended in molecular biology-grade water for measuring concentration

Step	Protocol	Incubations and notes
9. Library Preparation (Purple)	■ Transfer no more than **500 ng of DNA-containing Streptavidin Beads** to a fresh microcentrifuge tube. ■ Place the sample 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.
	■ To beads add: ■ 40 µL of **Molecular biology-grade water** ■ Cool to 4˚C, then add: ■ 4 µL of **Frag, End-repair, & A-Tail Buffer** ■ 6 µL of **Frag, End-repair, & A-Tail Enzyme**	Fragment, end-repair, and A-tail using thermocycler program listed in Step 9.
	■ To sample add: ■ 5 µL of **Universal Adapter** (diluted if necessary) ■ 20 µL **Adapter Ligation Mix**	Dilute Universal Adapter according to the table listed in Step 9.10. Incubate at 20˚C for 15 min, no heated lid.
	■ Wash the beads: ▸ Place the sample 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 150 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 4 washes.	
10. On-bead Amplification (Purple)	To beads add: ■ 20 µL of **molecular biology-grade water** ■ 25 µL **2X PCR Mix** ■ 5 µL of one **PCR Primer Mix**	Amplify with PCR program given in Step 10 of the detailed protocol.

Step	Protocol	Incubations and notes
11. Library Clean-up (Clean-up)	▪ Place the sample on a magnetic tube rack or magnet and allow solution to clear. ▪ Transfer 50 µL of the **library-containing supernatant** to a new tube.	
	▪ Add 57.5 µL of **Recovery Beads.**	Incubate at room temperature for 10 min.
	▪ Place the sample on a magnetic tube rack or magnet.	**Your library is in the supernatant. Do not discard.**
	▪ Transfer the supernatant (105 µL) to a new tube containing 15 µL of **Recovery Beads.**	Incubate at room temperature for 10 min.
	▪ Rinse the beads: ▸ Place the sample on a magnetic rack. ▸ Once the solution has cleared, remove 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.
	▪ Resuspend the beads in 30 µL of **Elution Buffer**.	Incubate at room temperature for 10 min.
	▪ Place the sample 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 12 in detailed Protocol for recommended QC to determine if your library is sufficient.

Detailed Protocol

1. Recommended Sample Pre-processing

PGShield™ treatment before crosslinking can contribute to improved library yield and quality for certain samples. While this quality improvement is not a guarantee, it does not typically negatively impact preparation of animal Hi-C Libraries.

1.1 Prepare sample according to the recommendation specific to your sample type as described in **Sample Types and Preparation.**

1.2 Remove any remaining buffer or media from sample, being careful to remove as much as possible.

Too much residual buffer or other liquid carryover can negatively impact your library prep.

1.3 Resuspend sample in **PGShield™**, approximately 2X volume **PGShield™** to sample volume.

*Sample inputs as recommended in **Sample Types and Preparation** typically require no more than 1 mL PGShield™, but this can vary per sample type. Make sure enough **PGShield™** is added so that the sample is fully covered, but not in extreme excess.*

1.4 Allow cells to sit in **PGShield™** for 1-12 hours at +2 to +8˚C.

*12 hours or more stored in **PGShield™** is recommended before proceeding, but 1 hour is sufficient to observe a positive impact. Samples can be stored in **PGShield™** at +2 to +8˚C for up to 1 year.*

2. Crosslinking (Red)

Prepare 1X CRB from 10X CRB by diluting with molecular biology-grade water.

2.1 If **1. Recommended Sample Pre-processing** was skipped, prepare sample according to the recommendation specific to your sample type as described in **Sample Types and Preparation**, then proceed to step 2.2.

2.2 If sample is still stored in **PGShield™** or some other storage solution, centrifuge at 10,000 *x g* for 5 min to pellet all sample material. Remove and discard the supernatant.

2.3 Resuspend sample in 1 mL of **Crosslinking Solution.**

*Make sure sample is completely covered and free-flowing in solution. If 1 mL of **Crosslinking Solution** is insufficient to achieve this, add an additional 500 µL of **Crosslinking Solution** to the sample.*

2.4 Incubate at room temperature for 20 min with occasional mixing by inversion or rotation.

2.5 Add 100 µL of **Quenching Solution**.

*If any additional **Crosslinking Solution** was added, use an additional 50 µL of **Quenching Solution**.*

2.6 Incubate at room temperature for 15 min with occasional mixing by inversion or rotation.

2.7 Centrifuge at 10,000 *x g* for 5 min to pellet all sample material. Remove and discard the supernatant.

***If working with blood that was not pre-processed with Red Blood Cell Lysis Buffer (not included), Crosslinking Solution** will lyse a large proportion of red blood cells resulting in a murky, red supernatant. White blood cells will form a faint, white pellet. Be careful to not disrupt the pellet while removing the dark supernatant.*

2.8 Wash the pellet with 1 mL of **1X CRB**, making sure the pellet has been fully disrupted and resuspended, then centrifuge at 10,000 *x g* for 1 min to gently compact the cellular material. Carefully remove and discard the supernatant.

To fully disrupt the pellet in 1X CRB, you do not need to be gentle. Vortex or use manual disruption as needed. Some sample types are more likely to stick to pipette tips, so proceed cautiously if mixing by pipetting.

SAFE STOPPING POINT: Crosslinked sample pellet can be stored at -25 to -15˚C for up to 1 month.

Proximo™ Hi-C Kit (Animal) Protocol v5.0

3. Cell Lysis (Orange)

Pre-heat a heating block, water bath, or thermocycler to 65˚C (for use in Step 3.10).
Bring Recovery Beads to room temperature.

3.1 Vortex **Lysis Buffer 1** to resuspend any particulates that may have settled out.

3.2 Resuspend the sample in 700 µL of **Lysis Buffer 1** and mix well by vortexing gently or pipetting thoroughly.

***If working with un-ground soft tissue,** transfer sample and 700 µL Lysis Buffer 1 to the loose fitting dounce. Slowly stroke the grinder 5-10 times being careful to minimize foaming. Proceed to Step 3.4.*

For all other sample types proceed to Step 3.3.

3.3 Incubate for 20 min at room temperature with occasional mixing by inversion or rotation.

3.4 Centrifuge at 500 *x g* for 1 min. **The chromatin is in the supernatant**.

***If working with cell pellets, blood, precious, or low-input sample**, skip steps 3.4-3.5 and move directly into Step 3.6 to help minimize sample loss.*

3.5 Carefully transfer the chromatin-containing supernatant to a fresh microcentrifuge tube.

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

3.6 Centrifuge the supernatant from Step 3.5 at 10,000 *x g* for 5 min and discard the supernatant. **The chromatin is now in the pellet**.

3.7 Optional: Wash pellet with **Lysis Buffer 1**.

This is recommended for samples with high fat content, adult insects, or any sample that typically performs poorly in standard DNA extractions. Otherwise proceed directly to Step 3.8

- Resuspend the pellet in 500 µL of **Lysis Buffer 1.**
- Centrifuge at 10,000 *x g* for 5 min
- Discard the supernatant.
- Repeat washes up to 3 times, or until supernatant is clear.

3.8 Resuspend the pellet in 500 µL of **1X CRB** and centrifuge at 10,000 *x g* for 5 min. Discard the supernatant.

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

3.9 Resuspend the pellet in 100 µL of **Lysis Buffer 2** and transfer the sample to a 0.2 mL PCR tube.

Make sure the pellet is fully covered by Lysis Buffer 2 and solution is free-flowing. If the pellet is too large, lysis efficiency may be diminished.

3.10 Incubate at 65˚C for 15 min.

3.11 Briefly allow sample to cool. Thoroughly resuspend **Recovery Beads** and add 100 µL of beads to sample. 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 6: Reverse Crosslinks**.*

3.12 Incubate at room temperature for 10 min.

3.13 Wash the beads:

- Place the sample 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 150 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 cleanly adhere to the magnet.

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

4. Fragmentation (Yellow)

Set up the following Fragmentation program:

Step	Temperature (˚C)	Time
Lid temperature	105	
Fragmentation	37	1 hr
Cool	4	Hold
Finishing	12	30 min
Final hold	4	Hold

4.1 Prepare **Fragmentation Mix**:

Reagent	Volume 1 reaction (µL)	Volume 8.2 reactions (µL)
Fragmentation Buffer	147.5	1209.5
Fragmentation Enzyme	2.5	20.5
Total	150	1230

4.2 Place the samples in a magnetic rack or on a magnet.

4.3 Once the solution in the tube has cleared, remove the supernatant without disrupting the beads.

4.4 Remove the tubes from the magnetic rack and gently resuspend the beads for each sample in 150 µL of prepared **Fragmentation Mix**.

4.5 Place the samples on the thermocycler and initiate the **Fragmentation program** run (Programmed at the beginning of Step 4. Fragmentation).

4.6 Once the samples have been through the Fragmentation step and cooled to 4˚C, keeping the samples on the thermocycler, open the lid and add 2.5 µL of **Finishing Enzyme** to each sample and mix by vortexing gently or pipetting thoroughly.

Proceed immediately into the Finishing Step. Do not allow your samples to hold between fragmentation and finishing for more than 5 minutes.

4.7 Progress the thermocycler program to the Finishing step.

4.8 Once the Finishing step is complete and samples are in the thermocycler at 4˚C, add 6 µL of **Stop Solution** to each sample, then remove from the thermocycler and mix by vortexing gently or pipetting thoroughly to quench the reaction.

*Promptly add **Stop Solution** after **Finishing Step** (30 minutes at 12°C). Extended incubation after the prescribed 30 minutes is complete may result in a low quality library.*

4.9 Wash the beads:

- Place the sample 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 150 µL of **1X CRB**.

4.10 Repeat the bead wash steps in Step 4.9 one more time with 150 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.

5. Proximity Ligation (Clear)

Set up the following **Ligation program**:

Step	Temperature (˚C)	Time
Lid temperature	105	
Ligation	25	4 hr
Enzyme inactivation	65	10 min
Final hold	4	Hold

5.1 Prepare **Ligation Mix:**

Reagent	Volume 1 reaction (µL)	Volume 8.2 reactions (µL)
Water	85	697
10X Ligation Buffer	10	82
Ligation Enzyme	5	41
Total	100	820

5.2 Place the samples in a magnetic rack or on a magnet.

5.3 Once the solution in each tube has cleared, remove the supernatant without disrupting the beads.

5.4 Remove the tubes from the magnetic rack and gently resuspend the beads for each sample in 100 µL of prepared **Ligation Mix**.

5.5 Place the samples on the thermocycler and initiate the **Ligation program** run (Programmed at the beginning of Step 5. Ligation**).**

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

6. Reverse Crosslinks (Clear)

Set up the following **Reverse Crosslinks** program:

Step	Temperature (˚C)	Time
Lid temperature	105	
Reverse crosslinks	65	1 hr
Final hold	4	Hold

6.1 Add 4 µL of **RX Enzyme** to each sample and mix well by vortexing or pipetting.

6.2 Place the samples on the thermocycler and initiate the run of the above **Reverse Crosslinks** program (Programmed at the beginning of **Step 6. Reverse Crosslinks**).

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

SAFE STOPPING POINT: The reaction may be stored at +2 to +8˚C overnight, or the supernatant can be removed and stored at -25 to -15˚C for up to 1 month.

7. Purify DNA (Green)

*Prepare **Recovery Wash Buffer** according to the instructions on the bottle.*

7.1 Allow sample to cool to room temperature.

7.2 Thoroughly resuspend the **Recovery Beads** and add 100 µL of **Recovery Beads** to the sample. Mix thoroughly by vortexing or pipetting.

*Recovering the supernatant from the used **Recovery Beads** before adding new **Recovery Beads** is acceptable but not necessary at this step. Removing used beads before the addition of new beads has not been found to notably impact the quality or yield of the final library for most sample types.*

7.3 Incubate at room temperature for 10 min.

7.4 Wash the beads:

- Place the sample 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 wash 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.

7.5 Repeat the bead wash steps in Step 7.4 once more for a total of 2 washes with **Recovery Wash Buffer**.

7.6 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**. As soon as beads look sufficiently dry, proceed to Step 7.7. Sample type variation may impact required drying time.*

7.7 Remove the sample from the magnet and thoroughly resuspend the beads in 100 µL of **Elution Buffer**.

7.8 Incubate at room temperature for 10 minutes to elute the DNA.

7.9 Place the sample in a magnetic tube rack or magnet.

7.10 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)

8. Streptavidin Bead Binding (Blue)

A. Prepare the Beads

Do not yet combine the beads with the DNA recovered in Step 7. DNA-binding will occur in section B after beads are prepared for binding.

8.1 Thoroughly resuspend the **Streptavidin Beads** and transfer 20 µL into a new microcentrifuge tube (or 0.2 mL PCR tube).

8.2 Wash the Beads:

- Place the sample 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 150 µL of **Wash Buffer 1**.

8.3 Repeat the bead wash steps one more time with 150 µL of **Wash Buffer 1** for a total of two washes.

8.4 Remove beads from the magnet and resuspend in 100 µL of **Bead Binding Buffer**.

B. Bind the Sample to the Beads

8.5 Transfer 100 µL of purified DNA (from Step 7) to the washed **Streptavidin Beads** (from Step 8.4) and mix by vortexing gently or pipetting thoroughly.

8.6 Incubate at room temperature for 10 min, mixing occasionally by gentle vortexing or inversion.

8.7 Wash the beads:

- Place the sample 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 150 µL of **Wash Buffer 2**.

8.8 Repeat the bead wash steps one more time with 150 µL of **Wash Buffer 2** for a total of two washes.

8.9 Repeat the bead wash steps one more time with 150 µL of **Wash Buffer 1**.

8.10 Repeat the bead wash steps one more time with 150 µL of **molecular biology-grade water.**

8.11 With your bead-bound sample suspended in 200 µL 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. To ensure an accurate measurement, vortex the beads in the fluorometric assay tube immediately prior to measuring DNA concentration.

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

Use between 2-5 µL for quantification. If at this stage the quantity of bead-bound DNA is less than 10 ng (0.5ng/µL concentration if using 2 µL for quantification), including "too low - out of range" measurements, this does NOT necessarily indicate failure, proceed to 9. Library Preparation. If you have any particular questions or concerns at this stage, we suggest reaching out to support@phasegenomics.com before proceeding.

SAFE STOPPING POINT: Store bead-bound sample at +2 to +8˚C overnight. Do not store longer than overnight before proceeding to **9. Library Preparation.**

Proximo™ Hi-C Kit (Animal) Protocol v5.0

9. Library Preparation (Purple)

Set up the following **Fragmentation, End-repair, and A-tailing** program:

Step	Temperature (˚C)	Time (min)
Lid temperature	105	
Pre-cooling	4	Hold
Fragmentation, end-repair, and A-tailing	30	7
	65	30
Final hold	4	Hold

And set up the following **Adapter Ligation** Program:

Step	Temperature (˚C)	Time (min)
Lid temperature	Off	
Adapter Ligation	20	15

9.1 Initiate the run of the **Fragmentation, End-repair, and A-tailing** program (Programmed at the beginning of Step 9. Library Preparation) to pre-cool the thermocycler.

9.2 Transfer no more than **500 ng of streptavidin-bound DNA** to a fresh microcentrifuge tube.

9.3 Place the sample in a magnetic rack or on a magnet.

9.4 Once the solution has cleared, remove and discard the supernatant without disrupting the beads.

9.5 Resuspend the beads in 40 µL of **molecular biology-grade water.**

9.6 Place the samples in the pre-cooled thermocycler and then cool to 4˚C for at least 1 min.

9.7 Prepare **FERAT Master Mix:**

Reagent	Volume 1 reaction (µL)	Volume 8.2 reactions (µL)
Frag, End-repair, A-Tail Enzyme	6	49.2
Frag, End-repair, A-Tail Buffer	4	32.8
Total	10	82

9.8 Add 10 µL of **FERAT Mix** to each sample and mix by vortexing gently or pipetting thoroughly. Immediately place the samples back onto the thermocycler.

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.

9.9 Advance the **Fragmentation, End-repair, and A-tailing** program from the Pre-cooling step to Fragmentation, end-repair, and A-tailing.

9.10 Every library for which the total mass of DNA measured at step 8.11 was less than 10 ng, prepare diluted **Universal Adapter** (provided tube is 15 µM) 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	5
0 - 10	1 µM	14	1

*Measured in Step 8.11

9.11 Add 5 uL of appropriately diluted **Universal Adapter** to each sample and mix by vortexing gently or pipetting thoroughly.

9.12 Add 20 µL of **Adapter Ligation Mix** to each sample. Mix by pipetting thoroughly.

Do not vortex Adapter Ligation Mix.

9.13 Place the samples on the thermocycler and initiate the run of the **Adapter Ligation** program (Programmed at the beginning of Step 9. Library Preparation)

Proximo™ Hi-C Kit (Animal) Protocol v5.0

9.14 Wash the beads:

- Place the sample 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 150 µL of **Wash Buffer 2**.

9.15 Repeat the bead wash steps one more time with 150 µL of **Wash Buffer 2** for a total of two washes.

9.16 Repeat the bead wash steps one more time with 150 µL of **Wash Buffer 1**.

9.17 Repeat the bead wash steps one more time with 150 µL of **molecular biology-grade water.**

10. On-bead Library Amplification (Purple)

Set up the following PCR Program:

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	

Use the table in step 10. 6 below to determine the number of PCR cycles to use for each sample.

10.1 Place the samples in a magnetic rack or on a magnet.

10.2 Once the solution in each tube has cleared, remove the supernatant without disrupting the beads.

10.3 Thoroughly resuspend the beads in 20 µL of **molecular biology-grade water**.

10.4 Add 5 µL 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).

10.5 Add 25 µL of **2X PCR Mix** and mix by vortexing gently or pipetting thoroughly.

Proximo™ Hi-C Kit (Animal) Protocol v5.0

10.6 Determine how many PCR cycles each of your samples requires according to the following table:

Mass used at step 8.11 (ng)	Recommended Number of PCR Cycles
<10	14
10-50	12
50-200	10
200-500	8

Amplifying your libraries beyond the suggested number of cycles can negatively impact the final data quality. This may require you to separate your samples into separate PCR runs

10.7 Place the samples on the thermocycler and initiate the run of the **PCR program** (Programmed at the beginning of Step 10. On-bead Library Amplification).

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

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

Use Recovery Wash Buffer Prepared at Step 7.

11.1 Place the sample in a magnetic tube rack or magnet.

11.2 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.*

11.3 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.

11.4 Incubate at room temperature for 10 min.

11.5 Place the sample in a magnetic tube rack or magnet. **Your library is in the supernatant. Do not discard.**

11.6 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. Make sure no beads from the first incubation transfer over with the supernatant.*

11.7 Incubate at room temperature for 10 min.

11.8 Wash the beads:

- Place the sample 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 wash 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.

11.9 Repeat the bead wash steps from Step 11.8 for a total of two washes 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.*

11.10 Remove the sample from the magnet and thoroughly resuspend the beads in 30 µL of **Elution Buffer**.

11.11 Incubate at room temperature for 5-10 min to elute the DNA.

11.12 Place the sample in a magnetic tube rack or magnet.

11.13 Once the solution has cleared, recover the **Proximo Hi-C Library-containing supernatant** and transfer to a fresh microcentrifuge tube. Discard the beads.

12. Library QC (recommended)

12.1 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.

12.2 Assess library fragment size using a BioAnalyzer or similar instrument.

The expected average library size is between 400-500 bp.

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).

13. Sequencing

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

Genome Size	Sequencing Recommendation
≤ 0.5 Gbp	≥ 50 million pairs (2 x 100 bp or longer)
0.5 Gbp – 1 Gbp	≥ 100 million pairs (2 x 100 bp or longer)
≥ 1 Gbp	100 Million pairs/Gpb (2 x 100 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.

14. Analysis

Before beginning any analysis workflow, we strongly recommend that you align your reads to a matching or closely related genome assembly to perform quality control. See our guide for instructions. Part of your QC process should include verifying that you have an appropriate number of usable Hi-C reads for your project.

Genome Phasing:
We strongly recommend that you use HiFi data along with your Hi-C data for assembly phasing. PacBio recommends 15x coverage per haplotype, so in order to phase a diploid genome, you will need 30x HiFi coverage. The Hi-C data requirements are equivalent to our scaffolding recommendations. If you have significantly more data than required, we do recommend downsampling the reads (try seqtk or bbtools reformat) to avoid choking the assembler. Use hifiasm with the Hi-C input option to produce phased draft assemblies. Then proceed to Genome Scaffolding!

Genome Scaffolding:
Contig-level or "draft" assemblies can be scaffolded using the ultra-long range contact information provided by Hi-C data. We recommend using the YAHS tool followed by manual curation in Juicebox (see tutorial here). We provide some tools for generating Juicebox-compatible files and for converting modified assemblies into fasta files in this repository. In the case of more complex or fragmented draft assemblies, you may also consider using the high-quality genome assembly of a closely related species for a reference-guided approach supported by Hi-C with a tool like RagTag. This should also be followed with manual Juicebox curation.

3D Genome Analysis:
If you are comparing multiple datasets for 3D genome analysis, we strongly recommend downsampling your datasets after QC to contain a comparable number of high-quality read pairs as the tools are sensitive to coverage. Note that for all 3D genome projects, it is critical to have a high-quality, chromosome-scale genome assembly.

For topologically associated domains (TADs), we recommend tools such as TopDom or Arrowhead. cLoops and HiCCUPS can identify chromatin loops in higher-resolution datasets.

Large-scale structural variants can be called using Hi-C Breakfinder.

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Index Sequences

Your kit contains two sets of indexed primers which are used to generate unique dual-indexed Illumina®-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 reference the **Phase Genomics Index Sequences** document, available at info.phasegenomics.com/protocols

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

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	• Corrected “SPRI” Spelling • Corrected “kit contents” volumes and tube numbers • Corrected pre-heat temperatures for “RX Crosslinking”
	2020-01	• 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	• 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	• 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	• 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. • Corrected inconsistent line weights in Kit Contents • Corrected Recovery Beads volume listed in Kit Contents

Proximo™ Hi-C Kit (Animal) Protocol v5.0

Version	Date	Revision Description
4.5	2022-12	• 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	• 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	• 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
4.5.2	2023-12	• Corrected discrepancies between quick and long protocol • Added more indexes and updated index IDs to new naming system • Converted font style • Updated PCR cycle guidelines • Updated adapter dilution guidelines • Updated index list to include more historical IDs
4.5.3	2024-04	• Increased fragmentation time from 5 to 7 min • Listed expected average fragment size in QC section

Version	Date	Revision Description
5.0	2026-08	• Decreased bead wash volumes from 200 uL to 150 uL • Updated sample input guidelines • Added PGShield™ use instructions • Updated contents to include PGShield™ • Added more in-line notes regarding sample preparation • Added dounce use instructions • Added master mix suggested volumes for 8 reaction volumes • Added thermocycler programs for fragmentation and setup instructions at the beginning of each step in which they are used • Expanded instructions to be useful for more than one kit variation • Increased base centrifugation speed from 6,000 to 10,000 *x g*. • Updated crosslink and quench time to sync up with other protocol versions • Updated sequencing recommendations • Adjusted formulation for Lysis Buffer 1 • Adjusted formulation for Crosslinking Solution and increased provided volume • Improved language consistency for bead washes and thermocycler protocol programming and use • Corrected PGShield™ trademark labeling and spacing. • Expanded analysis guidance • Updated workflow diagram • Merged multipack instructions into one unified protocol • Removed indexed adapter sequence information and moved to a separate document (Phase Genomics Index Sequences. Available at info.phasegenomics.com/protocols)

Proximo™ Hi-C Kit (Animal) Protocol v5.0

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