PHASE GENOMICS

Proximo™ Hi-C Kit

Proximo Hi-C (Fungal) Kit Protocol

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

This document applies to Proximo Hi-C (Fungal) Kit KT6045.

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.

Version 4.5

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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 microbiology.[4]

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 two dual-indexed Hi-C libraries from whole-cell fungal 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 fungal inputs (50 mg - 1 g). Any fungal sample type (from spores to filamentous fungi) 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 fungus requires additional preparation or reagents.

The Proximo Genome Scaffolding computational tool combines Hi-C sequencing data with draft short- or long-read assemblies to 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.

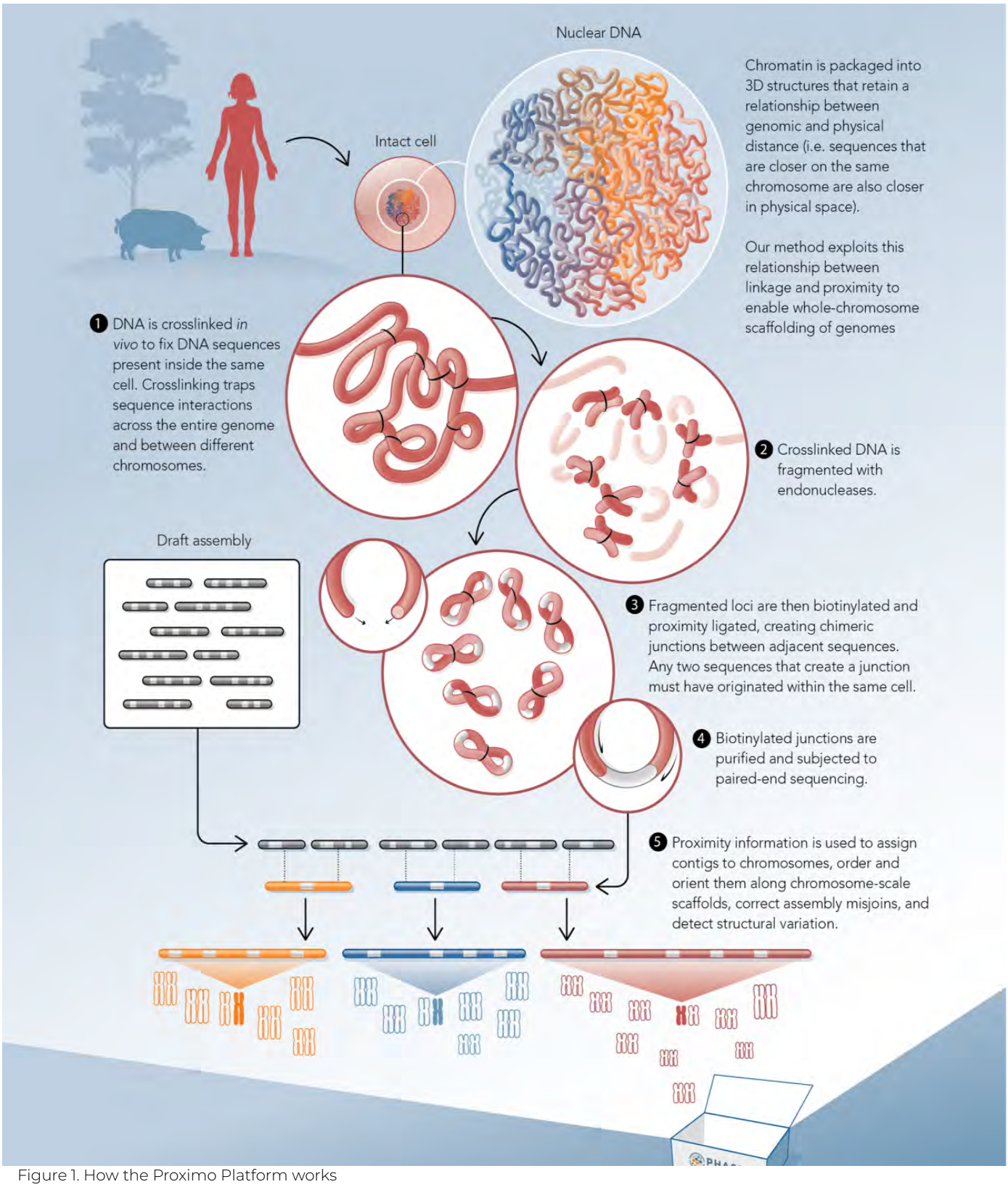

Figure 1. How the Proximo Platform works

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	Reference Code	Top Label	Tube Label	Volume per tube	No. of Tubes	Storage Temperature (˚C)	Used in Step	Before Starting
	KS0015	Crosslink Solution	Crosslinking Solution	1 mL	2	-25 to +8˚C	1.1	Thaw and warm to RT
	KS0003	Quench Solution	Quenching Solution	1 mL	1	-25 to +25˚C	1.3	Thaw and warm to RT[1]
	KB0036	Lysis Buffer 1	Lysis Buffer 1	1.4 mL	1	-25 to +25˚C	2.1-2.2	Thaw and warm to RT
	KB0003	Lysis Buffer 2	Lysis Buffer 2	500 µL	1	-25 to +25˚C	2.7	Thaw and warm to RT
	KC0001	Lysis Tube	Lysis Tube	500 µL	2	-25 to +25˚C	2.2	
	KB0006	Fragment Buffer	Fragmentation Buffer	300 µL	1	-25 to -15˚C	3.3	Thaw on ice
	KE0038	Fragment Enzyme	Fragmentation Enzyme	5 µL	1	-25 to -15˚C	3.4	Thaw on ice
	KE0016	Finishing Enzyme	Finishing Enzyme	6 µL	1	-25 to -15˚C	3.6	Thaw on ice
	KS0004	Stop Solution	Stop Solution	15 µL	1	-25 to -15˚C	3.8	Thaw and warm to RT
	KB0007	Ligation Buffer	Ligation Buffer	200 µL	1	-25 to -15˚C	4.2	Thaw on ice
	KE0026	Ligation Enzyme	Ligation Enzyme	4 µL	1	-25 to -15˚C	4.3	Thaw and warm to RT
	KE0007	RX Enzyme	RX Enzyme	10 µL	1	-25 to -15˚C	5.1	Thaw on ice
	KB0015	Elution Buffer	Elution Buffer	300 µL	1	-25 to +25˚C	6.7, 10.10	Thaw and warm to RT
	KR0011	Recovery Beads	Recovery Beads	600 µL	1	+2 to +8˚C	2.9, 6.2, 10.3, 10.6	Thaw and warm to RT
	KB0041	Recovery Wash Buffer	Recovery Wash Buffer	500 µL	1	+2 to +8˚C	6.5, 10.8	Warm to RT. Add 95%-100% Ethanol according to the instructions on the bottle.[2]
	KR0002	Strept Beads	Streptavidin Beads	40 µL	1	+2 to +8˚C	7.1	Thaw and warm to RT
	KB0012	Bead Bind	Bead Binding Buffer	250 µL	1	-25 to +25˚C	7.4	Thaw and warm to RT
	KB0047	Wash Buffer 1	Wash Buffer 1	7 mL	1	-25 to +25˚C	7.2, 7.3, 7.9, 8.15	Thaw and warm to RT
	KB0048	Wash Buffer 2	Wash Buffer 2	7 mL	1	-25 to +25˚C	7.7, 7.8, 8.13, 8.14	Thaw and warmx to RT
	KB0043	FERAT Buffer	Frag, Repair, A-tail Buffer	8 µL	1	-25 to -15˚C	8.6	Thaw on ice
	KE0029	FERAT Enzyme	Frag, Repair, A-tail Enzyme	12 µL	1	-25 to -15˚C	8.7	Thaw on ice
	KS0011	Universal Adapter	Universal Adapter	10 µL	1	-25 to -15˚C	8.9-8.10	Thaw on ice
	KE0032	Adapter Ligation	Adapter Ligation Mix	40 µL	1	-25 to -15˚C	8.11	Thaw on ice; do not vortex
	KE0011	HSR Mix	PCR Hot Start Ready Mix/ HSR Mix	50 µL	1	-25 to -15˚C	9.2	Thaw on ice
	KP000N[3]	Primer	Primer Mix	5 µL each	2	-25 to -15˚C	9.3	Thaw on ice
	KB0054	10X CRB	10X CRB	1.6 mL	1	-25 to -15˚C	1.6, 1.11, 2.6, 2.11, 3.7, 3.8	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

Shipping, Storage, and Handling

Proximo Hi-C (Fungal) 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 Library Reagent 1 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

Reagents for Optional Zymolyase Treatment

- Zymolyase (Zymo Research, Irvine, CA or similar)
- Phosphate-buffered saline, pH 7.4
- 2-mercaptoethanol (10 mM)

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
- 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 ≥6,000 ***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.

Sample Types and Preparation

This protocol is suitable for a wide range of fungal types, from spores to filamentous fungi.

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	Protocol Notes	Suggested Input
Spores or powder	During crosslinking, make sure that the solution is free-flowing after addition of Crosslinking Solution. If a sticky paste forms, increase added Crosslinking Solution up to 1.5 mL. Please reach out to support@phasegenomics.com if additional reagent is required.	50 mg - 200 mg
Filamentous fungi		100 mg - 1 g

Workflow Overview

Protocol Notes and Recommended QC

Quick Protocol

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

Step	Protocol	Incubations and notes
1. Crosslinking (Red)	▪ Grind sample to a fine powder with liquid nitrogen. ▪ Transfer tissue to a 2 mL microcentrifuge tube and add 1 mL of **Crosslinking Solution**.	Incubate at room temperature for 15 min while rotating.
	▪ Add 100 µL of **Quenching Solution**.	Incubate for room temperature for 20 min while rotating.
	▪ 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. ▪ Optional Zymolyase treatment. See detailed protocol (p. 17) for instructions.	
2. Lysis (Orange)	▪ Resuspend cells in 700 µL of **Lysis Buffer 1** and transfer to **Lysis Tube**.	Vortex for at room temperature for 20 min, using a bead-beater attachment if available.
	▪ Centrifuge for 10 sec in benchtop centrifuge.	
	▪ Transfer the supernatant to a clean microcentrifuge tube.	**The chromatin is in the supernatant.**
	▪ Centrifuge the supernatant at 6,000 *x g* for 5 min. ▪ Discard the supernatant.	**The chromatin is now in the pellet.**
	▪ 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˚C 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 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)	▪ 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**.	
	▪ 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 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)	▪ Remove **1X CRB** from beads. ▪ Add 85 µL **molecular biology-grade water.** ▪ Add 10 µL of **10X Ligation Buffer**.	
	▪ Add 5 µL of **Ligation Enzyme.**	Incubate at 20˚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)	▪ 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. Purifiy DNA (Green)	▪ Add 100 µL of **Recovery Beads** to the sample tube.	Incubate at room temp for 10 min.
	▪ Rinse the beads: ▸ 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** ▪ Remove **Recovery Wash Buffer** and air dry the beads.	To air dry, leave tubes with caps open on the magnet at room temperature for 5 - 15 min.
	▪ Resuspend the beads in 100 µL of **Elution Buffer**.	Incubate at room temperature for 5 min.
	▪ Place the sample tube on a magnetic tube rack or magnet. ▪ Once the solution has cleared, recover the **DNA-containing supernatan**t and transfer to a fresh tube.	

Step	Protocol	Incubations and notes
7. 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 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**.	
	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 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)	▪ 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.
	▪ To beads add: ▸ 40 µL of **Molecular biology-grade water** ▪ Cool to 4˚C, then add: ▸ 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.
	▪ 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 8.9. Incubate at 20˚C for 15 min, no heated lid.
	▪ Wash the beads: ▸ 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 4 washes.	
9. On-bead Amplification (Purple)	To beads add: ▪ 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)	▪ 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.	
	▪ Add 55 µL of **Recovery Beads.**	Incubate at room temperature for 10 min.
	▪ Place the sample tube 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 17.5 µL of **Recovery Beads.**	Incubate at room temperature for 10 min.
	▪ Rinse the beads: ▸ 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.
	▪ Resuspend the beads in 30 µL of **Elution Buffer**.	Incubate at room temperature for 5 min.
	▪ 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 Resuspend sample in 1 mL of **Crosslinking Solution.**

If you are working with spores, they may hydrate and form a paste at this step in the protocol. Add additional ***Crosslinking Solution*** *until the sample is free-flowing. This kit includes enough excess solution to add as much a 500 µL additional* ***Crosslinking Solution****. Please contact support@phasegenomics.com if additional reagent is required.*

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

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

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

1.5 Centrifuge at 6,000 *x g* for 5 min to pellet all sample material. Remove and discard the supernatant.

1.6 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 conpact the cellular material. Carefully remove and discard the supernatant.

1.7 Chill the sample pellet in liquid nitrogen or dry ice and grind to a fine powder. Proceed to 2. Cell Lysis if not performing the optional **Zymolyase** treatment.

Optional Zymolyase Treatment

1.8 Resuspend cells in 1 mL of **phosphate-buffered saline** pH 7.4 (not included) and 10 mM **2-mercaptoethanol** (not included and optional).

1.9 Add 5 U of **Zymolyase** (Zymo Research, Irvine, CA or similar, not included) and incubate for 1 h at 30°C.

1.10 Centrifuge at 6,000 *x g* for 5 min and discard the supernatant.

1.11 Resuspend the pellet with 1 mL of **1X CRB**.

1.12 Centrifuge at 6,000 *x g* for 5 min and discard the supernatant.

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

2. Cell Lysis (Orange)

Pre-heat a heating block, water bath, or thermocycler to 65˚C (for use in Step 2.8)

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

2.2 Resuspend cells in 700 µL of **Lysis Buffer 1** and add to **Lysis Tube.**

2.3 Vortex at room temperature for 20 min using a bead-beater attachment if available.

Other types of bead-beading shakers can be used. The appropriate duration and intensity will vary between instruments. Refer to manufacturer's recommendations.

However, 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.

2.4 Centrifuge at 500 *x g* for 10 sec to collapse bubbles and pellet debris, then transfer the supernatant to a clean microcentrifuge tube. **The chromatin is in the supernatant.**

For filamentous fungi or samples that have a lot of particulate carryover into the supernatant, *centrifuge at 500* x g *for 1 min and then transfer the supernatant to a new tube.*

2.5 Centrifuge the supernatant from Step 2.4 at 6,000 *x g* for 5 min and discard the supernatant. **The chromatin is now in the pellet**.

2.6 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 -15 to -25˚C for up to 1 month.

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

2.8 Incubate at 65˚C for 15 min.

2.9 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****.*

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

2.11 Incubate at 65˚C for 15 min.

2.12 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**.*

2.13 Incubate at room temperature for 10 min.

2.14 Wash the beads:

- Place the sample tube in 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**.

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 botttom 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)

3.1 Place the sample tube on a magnetic rack.

3.2 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.

4.1 Add 10 µL of **10X Ligation Buffer**.

4.2 Add 5 µL of **Ligation Enzyme** and mix by vortexing gently or pipetting thoroughly.

4.3 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 µL 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 µL of **Wash Buffer 1**.

7.3 Repeat the bead wash steps one more time with 200 µL of **Wash Buffer 1** for a total of two washes.

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

B. Bind the Sample to the Beads

7.5 Transfer 100 µL 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 µL of **Wash Buffer 2**.

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

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

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

7.11 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. 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.

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 uL 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 µL of **molecular biology-grade water**.

9.2 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).

9.3 Add 25 µL 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 55 µL (1.1X 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 17.5 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 µL 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 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 contact us at support@phasegenomics.com if additional indices or assistance with multiplexed sequencing are needed.

Index	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	AAAATTTC
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	AAGTGTCG
B4	ATGTGGAC	CTCACCAA	TTGGTGAG
C4	TGAGACGA	AACCAGAG	CTCTGGTT

Index	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
3.0	2020-01	· released
	2020-02	· tabulated incubation steps · expanded index table
4.0	2020-12	· Library Preparation and On-bead Library Amplification steps protocol re-formulated · Increased ligation buffer dilution factor for improved stability in long-term storage · 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 · Adjusted index sequence IDs to match 96 well layout · moved liquid nitrogen grinding from before the lysis step to before crosslinking · clarified brief centrifugation step · added clarifying comments to crosslinking and lysis steps
	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 Recovery Beads volume listed in Kit Contents
4.5	2022-12	· Added restriction enzyme information · clarified buffer removal before fragmentation · 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 · Removed duplicated Lysis steps · 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

Version	Date	Revision Description
	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

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