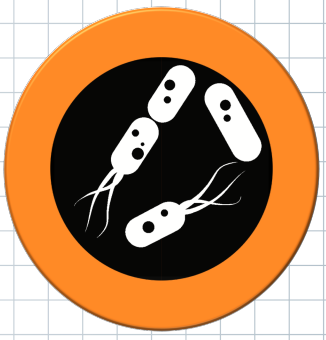
PHASE GENOMICS

Proximo™ Hi-C Kit

Version 3.0

Updated May 2020

Proximo (Microbe) Kit Protocol



For Crude-Sample Proximity Ligation Library Prep from Microbial Samples, for Illumina® Sequencing

This document applies to Proximo Hi-C (Microbe) Prep Kits KT1030.

Please read through this protocol before beginning your experiment. For customer support, please email support@phasegenomics.com or reference our Frequently Asked Questions.

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Kit Specifications

Kit Contents

Cap/label color	Ref. Code	Top label	Component name	Volume	Number of tubes	Storage temp. (ºC)	Used in Step	Before Starting
	KS0001	Crosslink Solution	Crosslinking Solution	1 mL	2	-25 to +25 ˚C	1.1	Thaw to RT
	KS0003	Quench Solution	Quenching Solution	1 mL	1	-25 to +25 ˚C	1.3	Thaw to RT*
	KB0036	Lysis Buffer 1	Lysis Buffer 1	1.4 mL	1	-25 to +25 ˚C	2.1-2.2	Thaw to RT
	KB0003	Lysis Buffer 2	Lysis Buffer 2	1 mL	1	-25 to +25 ˚C	2.7	Thaw to RT
	KC0001	Lysis Tube	Lysis Tube	500 µL	2	-25 to +25 ˚C	2.2	
	KB0005	Fragment Buffer	Fragmentation Buffer	300 µL	1	-25 to -15 ˚C	3.1	Thaw on ice
	KE0024	Fragment Enzyme	Fragmentation Enzyme	22 µL	1	-25 to -15 ˚C	3.2	Thaw on ice
	KB0007	Ligation Buffer	Ligation Buffer	200 µL	1	-25 to -15 ˚C	4.1	Thaw on ice
	KE0005	Ligation Enzyme	Ligation Enzyme	10 µL	1	-25 to -15 ˚C	4.2	Thaw to RT
	KE0007	RX Enzyme	RX Enzyme	10 µL	1	-25 to -15 ˚C	5.1	Thaw on ice
	KB0015	Elution Buffer	Elution Buffer	350 mL	1	-25 to +25 ˚C	6.8, 10.12	Thaw to RT
	KR0011	Recovery Beads	Recovery Beads	600 mL	1	+2 to +8 ˚C	2.9, 3.1, 4.1, 10.3, 10.6	Warm to RT
	KB0035	Recovery Wash Concentrate	Recovery Wash Concentrate	400 µL	1	+2 to +8 ˚C	6.6, 10.9	Warm to RT. Mix. 200 µL concentrate with 1 mL 95% Ethanol **
	KR0002	Strept Beads	Streptavidin Beads	40 µL	1	+2 to +8 ˚C	7.1	Warm to RT
	KB0012	Bead Bind	Bead Binding Buffer	250 µL	1	-25 to +25 ˚C	7.8	Warm to RT
	KR0001	Bead Reagent	Bead Reagent	20 µL	1	-25 to -15 ˚C	7.11	Thaw on ice
	KB0010	Wash Buffer 1	Wash Buffer 1	4 mL	1	-25 to +25 ˚C	7.4, 7.7, 7.19, 8.16	Thaw to RT
	KB0011	Wash Buffer 2	Wash Buffer 2	4 mL	1	-25 to +25 ˚C	7.15, 7.18, 8.12, 8.15	THaw to RT
	KB0009	Library Buffer	Library Buffer	50 µL	1	-25 to -15 ˚C	8.5	Thaw on ice
	KE0009	LR1/Library Reagent 1	Library Reagent 1	10 µL	1	-25 to -15 ˚C	8.6	Thaw on ice
	KS0005	Library Reagent 2	Library Reagent 2	10 µL	1	-25 to +25ºC	8.8	Thaw to RT
	KE0011	HSR Mix	PCR Hot Start Ready Mix/ HSR Mix	55 µL	1	-25 to -15 ˚C	9.2	Thaw on ice
	KP000N***	Index	Index Mix	5 µL each	2	-25 to -15 ˚C	9.3	Thaw on ice
	KB0017	10X CRB	10X CRB	1.6 mL	1	-25 to +25ºC	1.6, 1.11, 2.6, 2.13, 3.8, 3.11	Dilute to 1X before use.***

*Can heat at 37˚C to re-dissolve any precipitate that has crashed out after freezing
**Prepared Recovery Wash Buffer can be stored at +2 to +8˚C for up to 6 months
***1X CRB is stable stored at room temperature for up to 1 year

Shipping, Storage and Handling

Proximo Kits are shipped on cold packs. Upon receipt, remove inner container with Recovery 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 phasegenomics.com

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.

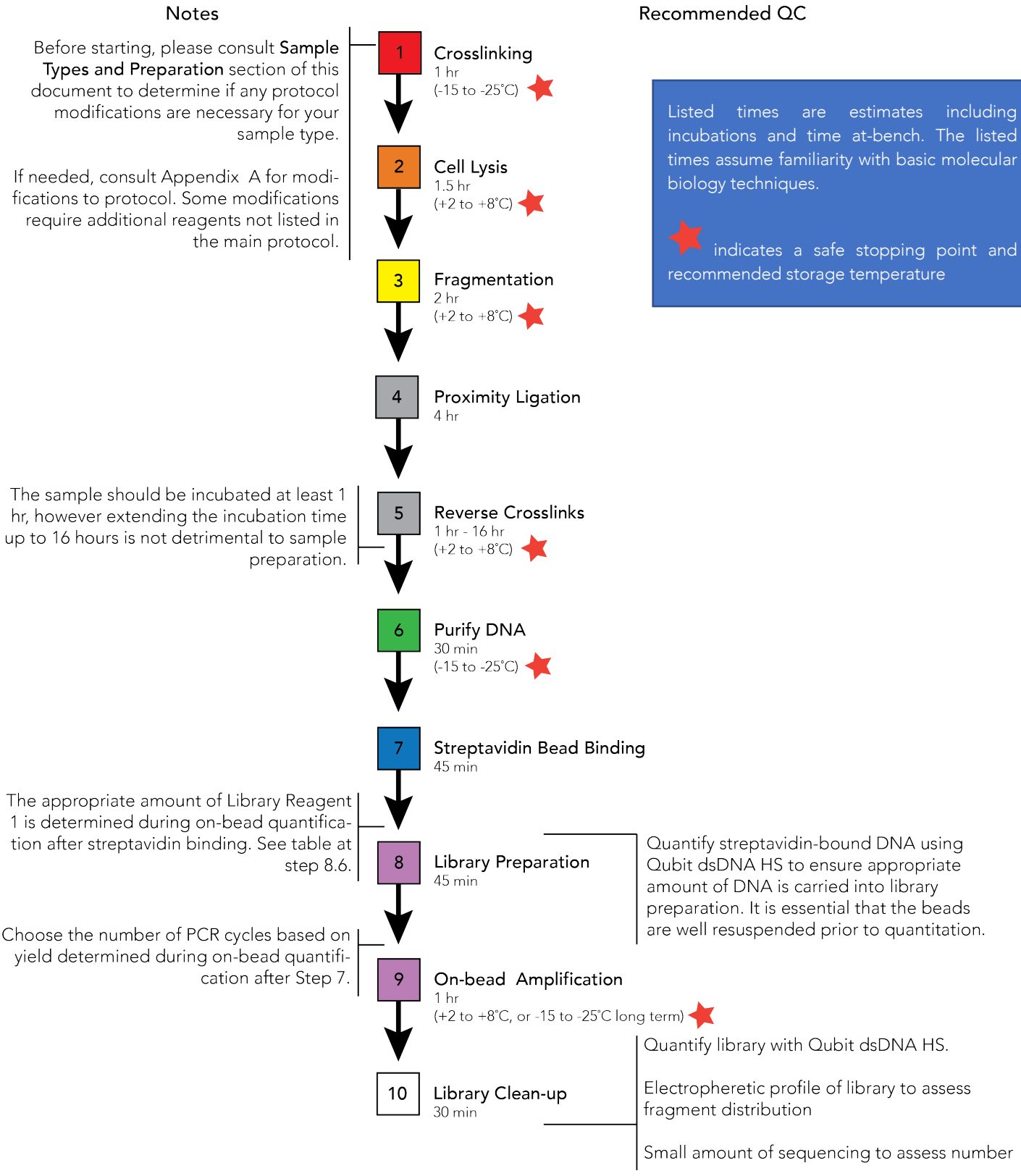
- 95% Ethanol
- Molecular biology-grade Water

Equipment and Consumables

No specialist equipment is required for this protocol. The following general laboratory equipment and consumables are needed.

- 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 2.8).
- Microcentrifuge capable of ≥17,000 x g
- Thermocycler
- Heating block or water bath that can maintain a temperature of 65˚C (alternatively use a thermocycler)
- Vortexer
- Qubit™ Fluorometer and Qubit dsDNA DNA HS Assay Kit (Thermo Fisher Scientific), or similar fluorometric assay for the quantification of double-stranded DNA

Workflow Overview



Sample Types and Preparation

The Proximo Kit protocol works with a wide variety of microbial and fungal sample types. Consult the table below for required inputs modifications to the standard protocol for you sample type.

Sample Type	Protocol Modifications	Input Requirements
Microbial Cell Pellet	None	1 – 20 million cells
Fecal Sample	None	50 – 100 µL
Soil	Crosslinking, see Appendix A-1 and A-2	Variable
Others (including low input)	Contact support@phasegenomics.com	Variable

Detailed Protocol

1 Crosslinking (Red)

See **Sample Types and Preparation** for sample input and possible protocol modification recommendations based on sample type.

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

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

1.3 Add 100 µL **Quenching Solution.**

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

1.5 Centrifuge at 17,000 *x g* for 5 min to pellet all sample material. Remove supernatant.

1.6 Wash the pellet with 1 mL **1X CRB** (prepared from included 10X CRB) and spin the microcentrifuge tube for 5 min at 17,000 *x g* to gently compact the cellular material. Carefully remove and discard the supernatant.

Perform 1 min 1,000 x g spin as needed to assist in removal of supernatant.

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

2 Cell Lysis (Orange)

See page 6 for possible lysis modifications based on sample type (soil, fungus, low-input).
Pre-heat a heating block, water bath, or thermocycler to 65˚C (for use in step 2.8)
Warm Recovery Beads to room temperature (for use in step. 2.9)

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

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

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

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

2.4 Centrifuge for 10 sec. in benchtop centrifuge to collapse bubbles and pellet debris, then transfer the supernatant to a clean 2 mL microcentrifuge tube. **The chromatin is in the supernatant.**

2.5 Centrifuge the supernatant from step 2.4 at 17,000 *x g* for 5 min and discard the supernatant. **The chromatin is now in the pellet.**

2.6 Resuspend the pellet in 500 µL **1X CRB** and centrifuge at 17,000 *x g* for 5 min. Discard the supernatant.

2.7 Resuspend the pellet in 100 µL **Lysis Buffer 2**.

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 beads to sample tube. Mix thoroughly by vortexing or by pipetting.

*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 Incubate at room temperature for 10 min.

2.11 Place the sample tube on a magnetic tube rack or magnet.

2.12 Once the solution has cleared, remove the supernatant without disrupting the beads.

2.13 Remove the tube from the magnet. Gently resuspend in 200 µL 1X CRB to wash.

SAFE STOPPING POINT:
Beads suspended in 1X CRB may be stored overnight at +2 to +8˚C.

2.14 Return the sample tube to the magnetic rack and wait for sample to clear.

Some samples may take a while to adhere to the magnet at this step. Just be patient, and careful when pipetting to remove the supernatant!

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

3 Fragmentation (Yellow)

Pre-heat thermocycler or heat block to 37˚C (for use in step 3.2)

3.1 Resuspend the beads fully in 150 µL **Fragmentation Buffer**.

3.2 Add 11 µL **Fragmentation Enzyme**, mix and incubate at 37˚C for 1 hr.

3.3 Return the sample tube to the magnetic rack and wait for sample to clear.

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

3.5 Remove the tube from the magnet. Gently resuspend the beads in 200 µL **1X CRB** to wash.

3.6 Return the sample tube to the magnet.

3.7 Once the Solution has cleared, remove and discard the supernatant without disrupting the beads.

3.8 Repeat steps 3.5 – 3.7 one more time with 200 µL **1X CRB** per wash, for a total of two washes.

SAFE STOPPING POINT: Store beads at +2 to +8˚C

4 Proximity Ligation (Clear)

4.1 Resuspend the pellet in 95 µL **Ligation Buffer**.

4.2 Add 5 µL **Ligation Enzyme** and mix.

4.3 Incubate the sample according to the following:

Step	Temperature (˚C)	Time
Ligation	20	4 hr
Enzyme Inactivation	65	10 min
	4	Hold

5 Reverse Crosslinks (Clear)

Pre-heat thermocycler or heat block to 65˚C (for use in step 5.2)

5.1 Add 5 µL **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 18 hours). **Your sample is now in the supernatant and no longer bound to the beads.**

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.
To prepare, add 1 mL 95% Ethanol to 200 µL provided Recovery Wash Concentrate and mix well.

6.1 Allow sample tube to cool to room temperature.

6.2 Resuspend **Recovery Beads** and add 100 µL **Recovery Beads** to the sample tube and mix thoroughly by vortexing or pipetting.

DNA has been released back into the supernatant during **5 Reverse Crosslinks**. More beads are added here to re-bind the DNA to the beads.

6.3 Incubate at room temperature for 10 min.

6.4 Place the sample tube on a magnetic tube rack or magnet.

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

6.6 Keeping beads on the magnet, gently wash beads twice with 200 µL **Recovery Wash Buffer** without disrupting the beads, leaving buffer on the beads for at least 30 sec between washes.

6.7 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****. They can be left drying for up to an hour, but should not need more than 10-15 minutes.*

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

6.9 Incubate for 10 min at room temperature to elute the DNA.

6.10 Place the sample tube on a magnetic tube rack or magnet.

6.11 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 -15 to -25˚C (indefinitely)

7 Streptavidin Bead Binding (Blue)

A. Prepare the Beads

7.1 Thoroughly resuspend the Streptavidin Beads and transfer 20 µL into a new 2 mL microcentrifuge tube (or 0.2 mL PCR tube).

7.2 Place the tube on a magnetic tube rack or magnet for at least 30 sec.

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

Do not yet combine the beads with the DNA recovered in Step 6.

7.4 Remove the tube from the magnet. Gently resuspend the beads in 200 µL **Wash Buffer 1**.

7.5 Return the sample tube to the magnet.

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

7.7 Repeat steps 7.4 – 7.6 one more time with 200 µL **Wash Buffer 1** per wash, for a total of two washes.

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

B. Bind the Sample to the Beads

7.9 Transfer 100 µL of purified DNA (from Step 6) to the **washed Streptavidin Beads** (from Step 7.8) and mix thoroughly by gentle vortexing or pipetting.

7.10 Incubate 5 min at room temperature, mixing occasionally by gentle vortexing or inversion.

7.11 Add 10 µL **Bead Reagent** and mix thoroughly by gentle vortexing or pipetting.

7.12 Incubate 5 min at room temperature, mixing occasionally by gentle vortexing or inversion.

7.13 Return the sample tube to the magnet.

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

7.15 Remove the tube from the magnet. Gently resuspend the beads in 200 µL **Wash Buffer 2**.

7.16 Return the sample tube to the magnet.

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

7.18 Repeat steps 7.15 – 7.17 one more time with 200 µL **Wash Buffer 2** per wash, for a total of two washes.

7.19 Repeat steps 7.15 – 7.17 one time with 200 µL **Wash Buffer 1**.

7.20 Remove the tube from the magnet. Gently resuspend the beads in 200 µL of **molecular biology-grade water**.

7.21 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.

8 Library Preparation (Purple)

Pre-heat thermocycler or heat block to 55˚C (for use in step 8.7)

8.1 Transfer no more than 50 ng of **DNA-containing Streptavidin Beads** to a fresh microcentrifuge tube.

Be sure to resuspend the beads thoroughly prior to transfer. Any remaining beads may be stored at +2 to +8˚C for subsequent analysis. If the amount of DNA measured in Step 7.21 is <50 ng, or even undetectable, proceed with the full 200 µL of beads from step 7.

Additionally, if the volume of beads transferred is less than 15 µL, allow additional time for the beads to adhere to the magnet and be very careful when removing the supernatant.

8.2 Place the sample tube on a magnetic tube rack or magnet.

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

8.4 Resuspend the beads in 20 µL of **molecular biology-grade water**.

8.5 Add 25 µL **Library Buffer**

8.6 Add the appropriate amount of **Library Reagent 1** and **molecular biology-grade water** as determined by the table below. Use no more than 50 ng sample. Mix by gentle vortexing or pipetting.

Total Sample Measured at Step 7.21	Library Reagent 1 Volume (µL)	Water volume (µL)
≤ 10 ng	1	4
10 – 20 ng	2	3
20 – 30 ng	3	2
30 – 40 ng	4	1
40 – 50 ng	5	0

8.7 Incubate at 55˚C for 10 min.

8.8 Add 5 µL **Library Reagent 2**. Mix by gentle vortexing or pipetting.

8.9 Incubate at 55˚C for 10 min.

8.10 Place the sample tube on a magnetic tube rack or magnet.

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

8.12 Remove the tube from the magnet. Gently resuspend the beads in 200 µL **Wash Buffer 2**.

8.13 Return the sample tube to the magnet.

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

8.15 Repeat steps 8.12 – 8.14 three more times with 200 µL **Wash Buffer 2** per wash, for a total of four washes.

8.16 Repeat steps 8.12 – 8.14 two times with 200 µL **Wash Buffer 1** per wash.

8.17 Repeat steps 8.12 – 8.14 one time with 200 µL **molecular biology-grade water**.

9 On-bead Library Amplification (Purple)

9.1 Resuspend the beads in 19 µL molecular biology-grade water.

9.2 Add 26 µL **HSR Mix** (**PCR Hot Start ReadyMix**).

9.3 Add 5 µL of one **PCR Index Mix** (use a different index for each sample. 2 unique indexes are provided with each kit. Sequences are found on page 23 of this document). Mix samples well by gentle vortexing or pipetting.

9.4 Amplify the library in a thermocycler programmed as follows:

Step	Temperature (˚C)	Time	Cycles
	72	5 min	1
Initial Denaturation	98	30 sec	1
Denaturation	98	10 sec	12*
Annealing	62	20 sec	
Extension	72	50 sec	
	4	Hold	1

**If less than 20 ng DNA was carried into step 8 (Library preparation), increase the number of PCR cycles to 15*

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

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

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. Discard the **Streptavidin Beads**.

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 are 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 5 min, or once the solution has cleared, transfer the supernatant (105 µL) to a new tube containing 17.5 µL **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 Place the sample tube on a magnetic tube rack or magnet.

10.9 Keeping beads on the magnet, gently wash beads twice with 200 µL **Recovery Wash Buffer** without disrupting the beads, leaving buffer on the beads for at least 30 sec between washes.

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

10.11 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**. They can be left drying for up to an hour, but should not need more than 10-20 minutes.*

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

10.13 Incubate for 5 min at room temperature to elute the DNA.

10.14 Place the sample tube on a magnetic tube rack or magnet.

10.15 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 of a successful library. The library can be stored at -15 to -25˚C

11.2 Assess library distribution using BioAnalyzer or similar instrument.

Acceptable sequencing-ready libraries have average distributions ranging between ~ 200 – 600 bp. If resulting library is outside that range, size selection is recommended.

Before performing a full sequencing run, it is recommended to sequence a small number of reads (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 tools (available at https://github.com/phasegenomics/hic_qc).

12 Sequencing

Proximo*f* Hi-C libraries are compatible with any illumina sequencer

Population Complexity	Sequencing Recommendation
Low- to mid-complexity communities (e.g. fecal microbiomes)	> 50 million pairs, paired-end 75 bp or greater for Hi-C > 100 million pairs, paired-end 150 bp for shotgun*
High-complexity communities (e.g. rumen, sludges, wastewater)	> 50 million pairs, paired-end 75 bp or greater for Hi-C > 200 million pairs, paired-end 150 bp for shotgun*

*>30X shotgun coverage is preferred for best results.

Need help with analysis?

Take a look at what we offer at
http://phasegenomics.com/technology/bioinformatics/
Or ask us about our analysis platform at info@phasegenomics.com

Index Primers

Included with the kit are Phase Genomics dual-unique indexes. Two dual-unique indexes are included with each Proximo Hi-C Kit but others are available at www.phasegenomics.com.

Index	i7 Equivalent Index	i5 Equivalent Index (For iSeq, NovaSeq, MiSeq, and HiSeq 2000/2500 sample sheet)	i5 Equivalent Index For MiniSeq, NextSeq, and HiSeq 3000/4000 sample sheet)
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
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

Appendix A

This appendix describes modifications to the protocol for soil and fungal samples. If you are unsure about the optimal preparation for your sample, please reach out to support@phasegenomics.com

1 Basic Soil Protocol

This protocol is designed for rich soils (i.e. top soils).

1.1 Mix 5 ml water per gram sediment on a vortexer or other strong mixer for 5 minutes. For rich soils, use 1 – 2 g of soil.

1.2 Spin tubes at 500 *x g* for 5 min to remove heavy particles.

1.3 Transfer supernatant to fresh microcentrifuge tube(s).

1.4 Centrifuge supernatant at 17,000 *x g* for 5 min.

1.5 Remove supernatant.

1.6 Proceed with pellet to step **1. Crosslinking.**

2 Low Abundance Soil Protocol

Differential Centrifugation of soil/sludge samples for Hi-C

This protocol is intended for soils with low levels of live organisms. For soils with abundant microbial life, follow the soil protocol included in ***Appendix A1****.*

Additional reagents not included in the kit are required.

2.1 Mix 5 mL water/g sediment on a vortexer or other strong mixer for 5 min.

2 tubes of 25 mL water + 5 g sediment is a good starting point.

2.2 Spin tubes at 1,000 *x g* for 10 min to remove heavy particles.

2.3 Recover the supernatant and add Formaldehyde to a final concentration of 1% (v/v)

2.4 Mix samples by inversion for 20 min at room temperature.

2.5 Add glycine to a final concentration of 1% (m/v) and mix the sample by inversion at room temp for 20 min.

2.6 Carefully layer 1 mL of supernatant on top of 1 mL of OptiPrep™ (or similar 60% iodixanol solution, not included).

Alternatively, you can layer larger volumes over the OptiPrep™ cushion if you have a centrifuge that can accommodate larger volumes.

It is also possible to sequentially load sups 1 mL at a time over the OptiPrep™ cushion.

2.7 Spin for 20 min at 10,000 *x g*.

2.8 Recover the supernatant and the cloudy interface and discard the lower fraction.

2.9 Spin the supernatant 17,000 *x g* at room temp for 5 min

2.10 Remove and discard the supernatant avoiding the pelleted cells.

2.11 Proceed to **2. Cell Lysis** with the pelleted cells.

Revision History

Document version	Date	Revision Description
2.0	June 2019	• Adjusted chemistry • Added reagents table • Added table of contents • Expanded required equipment and reagents • Added workflow overview • Reformatted protocol text • Added safe stopping points • Added pre-heat notes • Expanded on in-line notes • Added table for library reagent 1 volume addition • Decreased ligation volume • Retired indexes 1-8 • Corrected A1 index error
2.0.1	Sept 2019	• Added revision history • Adjusted fragmentation reaction conditions • Added more guidelines for bead clean-up post Reverse Crosslinks • Added note to bring SPRI beads to room temp before use • Added instructions to use water to bring library preparation reaction to 50 µL • Corrected spelling error of “nos” to “no.” on Notices page. • Added note to remove the supernatant before ethanol washes in final library clean-up
2.0.2	Oct 2019	• Corrected safe stopping point after Lysis to store pellet rather than store beads • Added bringing beads to RT for strept beads and SPRI in double-sided clean-up. • Added “discard supernatant” (step 10.9) • Added water volume column in table for “Library Prep” • Added “see sample table” guide at beginning of crosslinking and lysis • Added recommendation to not use additional mechanical lysis • Removed unnecessary “cool to 12˚C” from Fragmentation preparation instructions
	Jan 2020	• Removed index C1 • Added note about reheating Quench solution to re-dissolve crystals
3.0	Feb 2020	• Removed “Fungal Adaptation” in lieu of separate Fungal kit release • Switched from SPRI beads to Recovery Beads; component and volume change • 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 adjusted • 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 from storage of pellet at -20˚C. • General formatting update – font changed to match website, etc

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