

Feb 06, 2025

HiChIP Protocol using Arima-HiC+ Kits

DOI

dx.doi.org/10.17504/protocols.io.ewov1qq67gr2/v1

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DOI: dx.doi.org/10.17504/protocols.io.ewov1qq67gr2/v1

Protocol Citation: Anthony Schmitt, Jon Belton, Sara Tin, Jonathan Kirkland, Derek Reid, Xiang Zhou 2025. HiChIP Protocol using Arima-HiC+ Kits. [protocols.io https://dx.doi.org/10.17504/protocols.io.ewov1qq67gr2/v1](https://dx.doi.org/10.17504/protocols.io.ewov1qq67gr2/v1)

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Protocol status: Working

We use this protocol and it's working

Created: August 23, 2023

Last Modified: February 06, 2025

Protocol Integer ID: 86893

Keywords: Hi-C, HiChIP, PLAC-Seq, ChIP, ChiA-PET

Abstract

HiChIP Workflow Overview

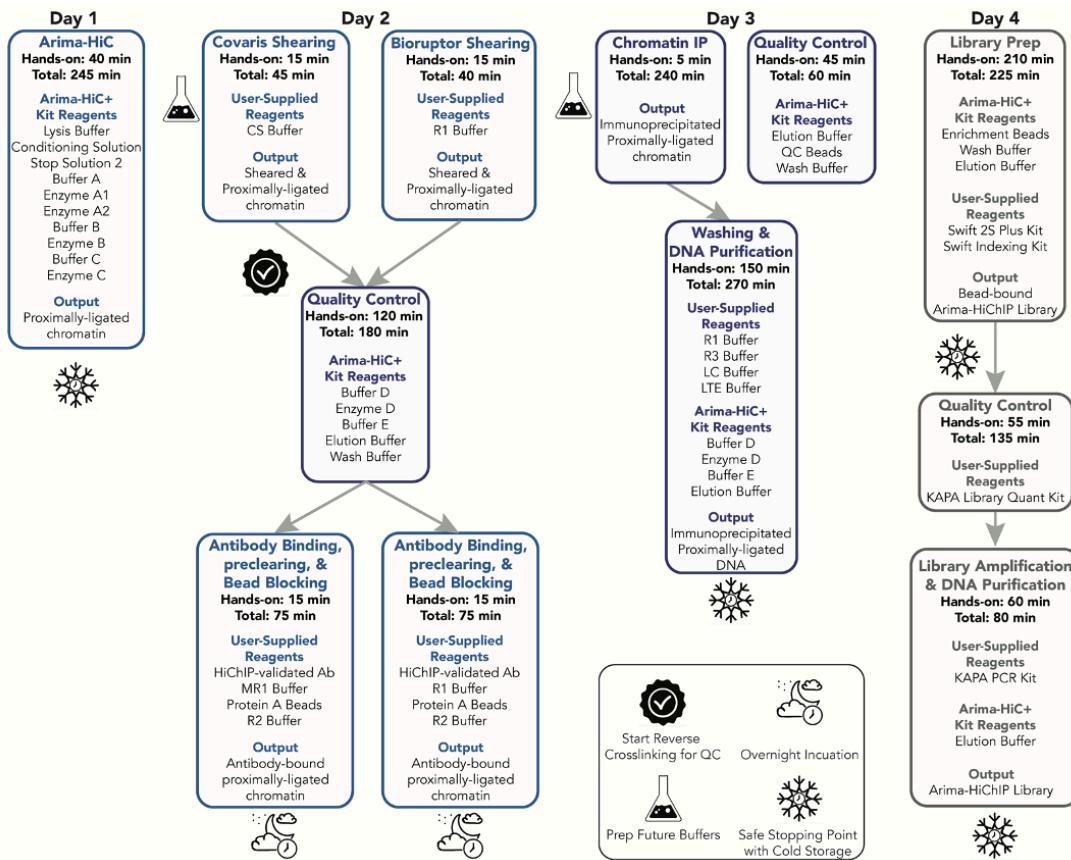
HiChIP is an experimental workflow that captures the structure (three-dimensional conformation) of genomes associated with a protein of interest. Chromatin from a sample source (e.g. cell lines) is first crosslinked to preserve the genome structure. The crosslinked chromatin is then digested using a restriction enzyme (RE) cocktail. The 5'-overhangs are then filled in with a biotinylated nucleotide. Next, spatially proximal digested ends of DNA are ligated, capturing the structure of the genome. The proximally-ligated chromatin is then sheared, bound to an antibody recognizing a protein of interest, immunoprecipitated, and purified to yield fragmented proximally-ligated DNA that was once bound *in vivo* to the protein of interest. The proximally-ligated DNA is subjected to a custom library preparation protocol utilizing a pre-validated commercially-available library prep kit. A separate HiChIP Library Prep user guide is provided that contains a custom protocol for enriching the proximally-ligated DNA for biotin and then converting the immunoprecipitated biotin-enriched DNA to HiChIP libraries.

Sequencing and Data Analysis

HiChIP libraries are sequenced via Illumina® sequencers in “paired-end” mode. The resulting data is referred to as HiChIP data. It is strongly recommended to process and analyze HiChIP using the publicly available tools Feather and MAPS (GitHub Link; Juric, 2019). This recommendation resulted from benchmarking analyses of computational tools for HiChIP data analysis and a collaboration to optimize the outputs of MAPS for maximum compatibility. However, other tools such as HiC-Pro (Servant, 2015) and FitHiChIP (Bhattacharyya, 2019) may also be used. All publicly available tools require usage modifications and custom input files that are specific to HiChIP data using Arima-HiC+ kits, so please carefully review. It is recommended to leverage the WashU Epigenome Browser (<http://epigenomegateway.wustl.edu>).

Guidelines

Workflow Overview



Workflow option 1. Streamlined Sample Processing. This option is best when the shearing conditions for the sample type on the given sonication platform have been determined previously in a HiChIP context. Note the experimenter can choose to stop at the safe stopping point at the end of day 1 or proceed into shearing and Antibody Binding.

Materials

Reagents, consumables, and equipment checklist:

- Arima-HiC+ Kit (Cat # A510008)
- Arima Library Prep Kit (Cat # A303011)
- 37% Formaldehyde (e.g. Fisher Scientific® Cat # F79–500)
- Ceramic mortar and pestle (e.g. Cole-Parmer® Cat # UX-63100–63)
- Metal spatula (Cole-Parmer® Cat # SI-06369–16)
- Cold-resistant gloves
- Dry Ice and Liquid Nitrogen
- Cell strainer, 40µm (CELLTREAT®, 229481)
- TLB1, TLB2, SS, CS Buffer, MR1 Buffer, R1 Buffer, R2 Buffer, R3 Buffer, LC Buffer, TE Buffer (see Section 2.4 and Appendices for recipes)
- 1M Tris-HCl, pH 8.0 (Fisher Scientific® Cat # 15–568–025)
- 5M NaCl (Sigma® Cat # S5150–1L)
- IGEPAL CO-630 (Sigma® Cat # 542334)
- Deionized Water (Fisher Scientific® Cat # LC267402)
- Sucrose (Sigma® Cat # S5016–25G)
- 1M MgAc (Sigma® Cat # 63052–100ML)
- 0.5M EDTA (Fisher Scientific® Cat # AM9260G)
- Triton X-100 (Sigma® Cat # T8787–50ML)
- 10% SDS (Fisher Scientific® Cat # MT-46040CI)
- 10% Sodium Deoxycholate (Fisher Scientific® Cat # 50–255–884)
- UltraPure™ BSA, 5mg/ml (Fisher Scientific® Cat # AM2616)
- 8M Lithium Chloride (Sigma® Cat # L7026–100ML)
- Protease Inhibitor Cocktail (Sigma® Cat # P8340–5ML)
- 1X PBS, pH 7.4 (e.g. Fisher Scientific® Cat # 50–842–949)
- Protein A Beads (Thermo Scientific® Cat # 10002D)
- Freshly prepared 80% Ethanol
- DNA Purification Beads (e.g. Beckman Coulter Cat # A63880)
- Qubit® Fluorometer, dsDNA HS Assay and tubes (Fisher Scientific Cat # Q32851, Q32856)
- 1.5mL, 15mL and 50mL tubes, including LoBind 1.5mL tubes (e.g. Genesee Cat # 86–923)
- PCR tubes (e.g. SSIbio® Cat # 3247–00) or PCR plates (e.g. Bio-Rad® Cat # HSS9641)
- Magnetic rack compatible with tube choice (e.g. Thermo Fisher Scientific® Cat # 12321D)
- Rotator, nutator, orbital shaker, or equivalent device for continuous mixing
- Centrifuge
- Thermal cycler (if performing parts of Arima-HiChIP in PCR tubes or PCR plate)
- Thermomixer
- Gel Electrophoresis System (e.g. Bioanalyzer®, TapeStation®, FlashGel™, etc.)

Workflow-specific user-supplied reagents, consumables, and equipment checklist

Tested Antibodies:

- H3K27Ac (Active Motif Cat # 91193 or 91194)
- H3K4me1 (Thermofisher Cat# 710795)
- H3K4me2 (Active Motif Cat# 39079 or 39679)
- H3K4me3 (Millipore Cat # 04–745)
- H3K79me2 (Millipore Cat# 04–835)
- CTCF (Active Motif Cat # 91285)
- POLII (Active Motif Cat# 39097 or 39497)
- Rad21 (Abcam Cat# ab992)

Chromatin shearing Instrument (either Diagenode® Bioruptor® Pico or Covaris®)

- Diagenode®: 0.65mL Microtubes for DNA Shearing (Diagenode® Cat # C30010011)
- Covaris®: microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm (Covaris® Cat # 520045)

Protocol materials

 Formaldehyde 37% Solution **Bio Basic Inc. Catalog #C5300-1.SIZE.1L** Step 2.4

 37% Formaldehyde **Fisher Scientific Catalog #F79-1** In 2 steps

 1x PBS **Fisher Scientific Catalog #BP243820** In 11 steps

Crosslinking – Cell Culture

- 1 **Input:** Cells collected from cell culture
Output: Crosslinked cells

Before you begin: This HiChIP workflow for mammalian cell lines begins with harvesting and crosslinking 12-15 million mammalian cells per biological replicate. Each reaction should only contain crosslinked cells comprising ~15µg of DNA (usually ~3-4 million mammalian cells), so this conservatively high quantity of cells recommended for crosslinking should be more than sufficient to complete the Estimating Input Amount and HiChIP Protocol. The excess cells account for less than expected DNA yields while also generating crosslinked cells for ChIP-seq experiments to define protein localization peaks. The crosslinking protocol below involves several cell pelleting centrifugations. For these centrifugations, pellet at the speed and duration you normally would for your specific cell type. Alternatively, we generally recommend centrifuging for 5 min at 1000 x G.

- 1.1 Harvest cells from cell culture using standard protocols and pellet cells by centrifugation. 
- 1.2 Resuspend cells in cell culture media, obtain a cell count by hemocytometer or automated cell counting methods. 
- 1.3 Transfer  12-15 million cells to be crosslinked into a new  15 mL conical tube, pellet cells by centrifugation and remove supernatant. 
- 1.4 Resuspend cells in  5 mL of  Room temperature  1x PBS Fisher Scientific Catalog #BP243820 . 
- Note:** In the below step, add methanol-stabilized formaldehyde to crosslink cells at a final formaldehyde concentration of 2%. Please DO NOT use other formaldehyde concentrations.
- 1.5 Add  286 µL of  37% Formaldehyde Fisher Scientific Catalog #F79-1, to bring the final formaldehyde concentration to 2%. 
- 1.6 Mix well by inverting 10 times and incubate at  Room temperature for  00:10:00 . 


1.7 Add  460 μL of Stop Solution 1, mix well by inverting 10 times and incubate at

 Room temperature for  00:05:00.

5m



1.8 Pellet cells by centrifugation.



1.9 Discard supernatant.

1.10 Resuspend cells in  1 mL  1x PBS Fisher Scientific Catalog #BP243820.



1.11 Aliquot cells into several new  1.5 mL tubes, two with 0.5  0.5 million cells per aliquot, and one with the remaining  11-14 million cells. Mix sample by inversion between aliquots.



1.12 Pellet cells in all aliquots by centrifugation.



1.13 Discard supernatant, leaving only the crosslinked cell pellets and no residual liquid.

1.14 Freeze samples on dry ice or liquid nitrogen, and store at  -80 $^{\circ}\text{C}$ until ready to proceed to the *Estimating Input Amount* protocol the following section.



Crosslinking – Cryopreserved Cells

2 **Input:** Cryopreserved cells

Output: Crosslinked cells

Before you begin: We recommend that the Arima-HiChIP workflow for mammalian cell lines begin with the crosslinking of cells harvested from cell culture, however, under certain circumstances one can also crosslink cells preserved in a cryogenic “freeze” media such as a mixture of complete cell culture media, FBS, and DMSO. A typical example would be cells that were once cultured and then collected at 5 million cells per mL in cryogenic “freeze” media, and stored in a liquid nitrogen tank. The crosslinking protocol below involves several cell pelleting centrifugations. During these centrifugations, pellet your specific cell types at a speed and duration as you normally would. Alternatively, we generally recommend centrifuging for 5 min at 500 x G.

2.1 Fill a  15 mL conical tube with  4 mL of  1X PBS (Phosphate-buffered saline) . 

2.2 Thaw the cryopreserved cells in a  37 °C water bath. 

Note: In the following step, the entire contents of the cryopreserved cell sample (i.e. cells and the cryogenic media) are transferred into the conical tube containing

 1X PBS (Phosphate-buffered saline) . Do not centrifuge the cells to try and remove the cryogenic freeze media. The following step also assumes the cells are preserved in  1 mL of cryogenic freeze media, and transferring the cells into the  1X PBS (Phosphate-buffered saline) will bring the total volume to  5 mL . If the cells are not frozen in  1 mL of cryogenic freeze media, adjust the volume of  1X PBS (Phosphate-buffered saline) so that the total sample volume after Step 3 will be  5 mL .

2.3 Gently transfer cells, including the cryogenic freeze media, into the conical tube containing  4 mL of  1X PBS (Phosphate-buffered saline) , bringing the total volume to  5 mL .

2.4 Add  286 µL of  Formaldehyde 37% Solution **Bio Basic Inc. Catalog #C5300-1.SIZE.1L** , bringing the final formaldehyde concentration to 2%.

2.5 Mix well by inverting 10 times and incubate at  Room temperature for  00:10:00 



2.6 Add  460 µL of Stop Solution 1, mix by inverting 10 times and incubate at  Room temperature  00:10:00 . 



2.7 Place sample at  On ice and incubate for  00:15:00 . 

15m



2.8 Pellet cells by centrifugation and discard supernatant. 



- 2.9 Resuspend cells in 1 mL 1X PBS (Phosphate-buffered saline).
- 2.10 Aliquot cells into several new tubes, two with 0.5 million cells per aliquot, and one with the remaining 11-14 million cells. Mix sample by inversion between aliquots.
- 2.11 Pellet cells in all aliquots by centrifugation.
- 2.12 Discard supernatant, leaving only the crosslinked cell pellets and no residual liquid.
- 2.13 Freeze samples on dry ice or liquid nitrogen, and store at -80 °C until ready to proceed to the Estimating Input Amount protocol the following section.

Crosslinking – Large Animal Tissue

- 3 **Input:** Fresh-frozen large animal tissue
Output: Crosslinked nuclei

Before you begin: The Arima-HiChIP workflow for large animal tissues begins with the pulverization and crosslinking of fresh-frozen large animal tissue. For most vertebrates and large invertebrates that comprise dense tissues, begin by weighing 200-600mg of fresh frozen tissue, and record this measured mass. The measured mass will be used later in this protocol and the following Estimating Input Amount – Large Animals protocol. For some applications, less than 200mg can be used, particularly when sample quantity is scarce. Note that this crosslinking protocol requires the handling of liquid nitrogen, dry ice, and severely cold equipment. Please use extra caution and wear cold-resistant gloves and appropriate PPE as needed.

- 3.1 Prepare buffers TLB1, TLB2, SS using the recipes in Appendix A and cool on ice. Also, cool a centrifuge large enough to spin 50 mL conical tubes at 4 °C and cool 10 mL of 1x PBS Fisher Scientific Catalog #BP243820 on ice per sample.
- 3.2 Embed a mortar, with a pestle inside of it, and a 50 mL conical tube onto a cooler of dry ice and allow to cool. Cool a spatula by placing it in the 50 mL conical tube for later use.

- 3.3 Pour liquid nitrogen into the mortar until the entire pestle tip is submerged. Allow liquid nitrogen to evaporate completely to cool the mortar and pestle.
- 3.4 Pour liquid nitrogen into the mortar until the entire pestle tip is submerged. Transfer  200-600 mg frozen large animal tissue into mortar containing liquid nitrogen. Allow liquid nitrogen to evaporate just enough for the tissue to stay submerged.



- 3.5 Pulverize the tissue in the mortar using the pestle until the sample resembles a fine powder like corn starch or powdered sugar. Ensure the tissue is always submerged in liquid nitrogen. Carefully re-fill the mortar with liquid nitrogen as necessary. The pulverization process should take at least  00:05:00 per sample and some tissue types may take longer. The goal is to pulverize until the tissue resembles a fine powder without visible chunks.

5m



- Note:** Using cold-resistant gloves is highly recommended to handle the mortar and pestle.
- 3.6 Once the sample resembles a fine powder, allow liquid nitrogen in the mortar to evaporate just enough for sample to stay submerged in a viscous slurry.
 - 3.7 Carefully pour pulverized tissue and remaining liquid nitrogen from the mortar into the  50 mL conical tube . Ensure the tube does not overflow with liquid nitrogen.
 - 3.8 Using the cooled spatula from Step 2, transfer any remaining pulverized large animal tissue from the mortar into the  50 mL conical tube .

- 3.9 Submerge  50 mL conical tube into dry ice to keep all the pulverized animal tissue frozen.



- Note:** DO NOT cap the  50 mL conical tube until all the liquid nitrogen has completely evaporated.
- 3.10 Allow liquid nitrogen in  50 mL conical tube to evaporate completely.



- Note:** If pulverizing multiple animal tissue samples in a single day, keep the pulverized sample on dry ice and repeat Steps 1-9 on the remaining samples using clean equipment and consumables until all samples have been pulverized. Then, proceed to the next step and complete the remaining protocol on all samples simultaneously.
- 3.11 Remove sample tube from dry ice.

- 3.12 Incubate sample tube for  00:10:00 on ice. 10m 
- 3.13 Add  5 mL of cold  1x PBS Fisher Scientific Catalog #BP243820 and resuspend via pipetting with a 1ml pipette. 
- 3.14 Pellet sample by centrifugation at  1.000 x g at  4 °C for  00:05:00. 5m 
- 3.15 Discard supernatant.
- 3.16 Add  3 mL of TLB1 and resuspend via pipetting with a 1ml pipette. 
- 3.17 Incubate sample tube for  00:20:00 at  4 °C, occasionally mix by inverting. Proceed to steps 18 and 19 below during the incubation. 20m 
- 3.18 Prepare a fresh  50 mL conical tube with a 40µm cell strainer on top, per sample.
- 3.19 Rinse the 40µm cell strainer with  5-10 mL DI water, discard flow through.
- 3.20 Add suspended sample to the cell strainer and allow the liquid to drip into the  50 mL conical tube. Stir gently with a clean spatula to assist flow through. Continue stirring until most of the liquid has flowed through the filter.
- 3.21 Rinse cell strainer with  2 mL of TLB2, stir with spatula until most of the liquid flows through.

Note

Some minor residual liquid will remain in cell strainer. Gentle stirring will help prevent the strainer from clogging.

3.22 Tap the cell strainer on the top of the  50 mL conical tube to dislodge any remaining drips of liquid. Discard the cell strainer and cap the tube. Ground tissue that is less than 40 µm will be in the  50 mL conical tube. This will include small cell clumps, single cells and nuclei.

3.23 Pellet sample by centrifugation at  1.000 x g, 4°C for  00:05:00. Reduce the slowdown speed of the centrifuge so that the pellet will not be disturbed when the centrifuge stops.

5m



°C

3.24 Discard supernatant, making sure not to disturb the pellet.

3.25 Resuspend sample in  1 mL of TLB2, using a 1ml pipette.



3.26 Overlay  3 mL of buffer SS (Sucrose Solution) carefully using a 5ml serological pipette by pipetting no faster than 0.1ml per second. The SS should form a layer on top of the cell suspension.

3.27 Pellet sample by centrifugation at  2.500 x g, 4°C, 00:05:00 for  00:05:00. Reduce the slowdown speed of the centrifuge so that the sucrose gradient will not be disturbed when the centrifuge stops.

10m



°C

3.28 Discard supernatant.

3.29 Resuspend sample in  1 mL of cold  1x PBS Fisher Scientific Catalog #BP243820

3.30 Pellet sample by centrifugation at  2.500 x g, 4°C, 00:05:00 for  00:05:00.

10m



°C

3.31 Discard supernatant.

Note

In the below step, add formaldehyde to crosslink cells at a final formaldehyde concentration of 2%. Please DO NOT use other formaldehyde concentrations.

3.32 Resuspend sample in  5 mL of room temperature

 1x PBS Fisher Scientific Catalog #BP243820 .

3.33 Add  286 μ L of  37% Formaldehyde Fisher Scientific Catalog #F79-1 , to bring the final formaldehyde concentration to 2%. 

3.34 Mix well by inverting 10 times and incubate at  Room temperature for  00:05:00 . Mix the sample every 2-3 mins with gentle inversion. 

3.35 Add  460 μ L of Stop Solution 1, mix well by inverting 10 times and incubate at  Room temperature for  00:05:00 . Mix the sample every 2-3 mins with gentle inversion.  

3.36 Place sample on ice and incubate for  00:15:00 . 



3.37 Pellet sample by centrifugation at  2.500 x g, 4°C, 00:05:00 . 



3.38 Discard supernatant.

3.39 Resuspend sample in  3 mL cold  1x PBS Fisher Scientific Catalog #BP243820 . 

3.40 Pellet sample by centrifugation at  2.500 x g, 4°C, 00:05:00 . 



3.41 Discard supernatant.

- 3.42 Resuspend sample in  1 mL cold  1x PBS Fisher Scientific Catalog #BP243820 , and transfer to a  1.5 mL microfuge tube . 
- 3.43 To prepare for the Estimating Input Amount – Large Animals protocol in a following section, mix the sample by pipetting and then immediately aliquot sample such that 2 aliquots (DI Aliquots) contains the equivalent of 10mg of the original pulverized large animal tissue, while the rest of the aliquots each contain the equivalent of ~20-25% (Storage Aliquots) of the pulverized large animal tissue. Mix sample by inversion between aliquots to ensure all aliquots are equally homogeneous. For example, if processing 200mg of pulverized large animal tissue, then take 5% of the suspended material. The remaining aliquots containing 20-25% are meant to be saved as sample material for the Arima-HiC Protocol.
- 3.44 Pellet all aliquots by centrifugation at  2.500 x g, 4°C, 00:05:00 . Discard supernatant leaving behind only the sample pellet and no residual liquid. 
- 3.45 Snap freeze the aliquot cell pellets on dry ice for  00:05:00 or liquid nitrogen for  00:01:00 , then store at  -80 °C for up to one year. 

Estimating Input Amount – Mammalian Cell Culture and Cryopreserved Cells

- 4 **Input:** Crosslinked cells
Output: Purified genomic DNA

Before you begin: Arima-HiChIP reactions are optimally performed on crosslinked cells comprising 15µg of DNA. This amount of DNA ensures efficient Arima-HiC biochemistry, optimal chromatin immunoprecipitation performance, and sufficient Arima-HiChIP library complexity for the validated histone modification or transcription protein targets. The Estimating Input Amount protocol is required if one does not know how many crosslinked cells will comprise 15µg of DNA. The Estimating Input Amount protocol measures the amount of DNA obtained per 0.5 million crosslinked cells, in duplicate, and uses this to estimate the optimal cellular input for an Arima-HiChIP reaction. The Arima-HiC+ kit contains sufficient reagents to perform the Estimating Input Amount protocol on up to 8 samples, in duplicate, totaling up to 16 Estimating Input Amount reactions if needed. This protocol should be performed in microfuge tubes. This section concludes with a descriptive example of how to estimate the optimal number of crosslinked cells to use per Arima-HiChIP reaction.

- 4.1 Thaw the two  0.5 million cell aliquots prepared during the Crosslinking protocol.

Note

The remaining protocol steps are meant to be applied to both replicates from each sample in order to obtain a more robust estimation of DNA yield per 0.5 million cells. Also note Step 2 requires addition of several reagents in the same step. These reagents should be combined into master mixes with 10% excess volume before use.

- 4.2** Add 209.5 µL of a master mix containing the following reagents:



| Reagent | Volume per reaction | x 10% extra | x # of reaction | Final |
|----------------|---------------------|-------------|-----------------|----------|
| Buffer D | 174 µL | 191.4 µL | 2 | 382.8 µL |
| Elution Buffer | 10.5 µL | 11.55 µL | 2 | 23.1 µL |
| Enzyme D | 25 µL | 27.5 µL | 2 | 55 µL |
| Total | 209.5µL | | | 460.9µL |

- 4.3** Add 20 µL of Buffer E, mix gently by pipetting, and incubate at 55 °C for 00:30:00 .

30m



- 4.4** Incubate at 68 °C for at least 03:00:00 .

3h

**Note**

To provide flexibility in the workflow, this incubation can be held overnight if a thermal cycler or thermomixer with a heated lid is used to prevent evaporation inside the tube.

- 4.5** Incubate at Room temperature for 00:10:00 .

10m

**Note**

DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to RT and thoroughly mixed before use. The DNA Purification Beads are a user-supplied reagent and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC+ kit.

- 4.6 Add $\text{225 } \mu\text{L}$ of DNA Purification Beads, mix thoroughly, and incubate at Room temperature for $00:05:00$.
- 4.7 Place sample against magnet, and incubate until solution is clear.
- 4.8 Discard supernatant. While sample is still against magnet, add $\text{500 } \mu\text{L}$ of freshly prepared 80% ethanol, and incubate at Room temperature for $00:01:00$.
- 4.9 Discard supernatant. While sample is still against magnet, add $\text{500 } \mu\text{L}$ of 80% ethanol, and incubate at Room temperature for $00:01:00$.
- 4.10 Discard supernatant. While sample is still against magnet, incubate beads at Room temperature for 3-5 min. to air-dry the beads.
- 4.11 Remove sample from magnet, resuspend beads thoroughly in $\text{50 } \mu\text{L}$ of Elution Buffer, and incubate at Room temperature for $00:05:00$.
- 4.12 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
- 4.13 Quantify sample using a Qubit following the manufacturer's instructions. Use $\text{2 } \mu\text{L}$ of DNA for the Qubit assay. The total DNA yield corresponds to the amount of DNA obtained from 0.5 million mammalian cells.
- 4.14 Estimate how many mammalian cells to use per Arima-HiChIP reaction. See the example below:

Example: We recommend aiming to use crosslinked cells comprising $15 \mu\text{g}$ of DNA per Arima-HiChIP reaction. If an average of $2 \mu\text{g}$ of DNA was obtained per 0.5 million mammalian cells as calculated in Step 13, one can estimate that 3.75 million crosslinked cells should be used per Arima-HiChIP reaction ($15 \mu\text{g}$ of DNA). Additionally, please note that the crosslinked cell pellet for one Arima-HiChIP reaction should occupy no more than

20 µL of volume in the sample tube. If the crosslinked cell pellet comprises 15 µg of DNA but occupies greater than 20 µL of volume, aliquot the cells into multiple Arima-HiChIP reactions such that the sum of the DNA input from all reactions is 15 µg and each cell pellet occupies no more than 20 µL of volume, or contact Technical Support for additional guidance.

Estimating Input Amount – Large Animal Protocol

5 **Input:** Pulverized Crosslinked Tissue

Output: Purified genomic DNA

Before you begin: Arima-HiChIP reactions are optimally performed on crosslinked cells comprising 15 µg of DNA. This amount of DNA ensures efficient Arima-HiC biochemistry, optimal chromatin immunoprecipitation performance, and sufficient Arima-HiChIP library complexity for the validated histone modification or transcription protein targets. The Estimating Input Amount protocol is required if one does not know how many crosslinked cells will comprise 15 µg of DNA. The Estimating Input Amount – Large Animal protocol measures the amount of DNA obtained per 10 mg of unprocessed tissue, in duplicate, and uses this to estimate the optimal input amount for an Arima-HiChIP reaction. The Arima-HiC+ kit contains sufficient reagents to perform the Estimating Input Amount protocol on up to 8 samples, in duplicate, totaling up to 16 Estimating Input Amount reactions if needed. This protocol should be performed in microfuge tubes. This section concludes with a descriptive example of how to estimate the optimal amount of input material to use per Arima-HiChIP reaction.

5.1 Thaw the two 10 mg DI Aliquots prepared during the **Crosslinking – Large Animal protocol.**

Note

The remaining protocol steps are meant to be applied to both replicates from each sample in order to obtain a more robust estimation of DNA yield per 10mg of input material. Also, note Step 2 requires addition of several reagents in the same step. These reagents should be combined into master mixes with 10% excess volume before use.

5.2 Add 209.5 µL of a master mix containing the following reagents:



| Reagent | Volume per reaction | x 10% extra | x # of reactions | Final |
|----------------|---------------------|---------------|------------------|---------------|
| Elution Buffer | 174 μ L | 191.4 μ L | 2 | 382.8 μ L |
| Buffer D | 10.5 μ L | 11.55 μ L | 2 | 23.1 μ L |
| Enzyme D | 25 μ L | 27.5 μ L | 2 | 55 μ L |
| Total | 209.5 μ L | | | 460.9 μ L |

5.3 Add  20 μ L of Buffer E, mix gently by pipetting, and incubate at  55 °C for

 00:30:00

30m



5.4 Incubate at  68 °C for at least  03:00:00 .

3h



Note

To provide flexibility in the workflow, this incubation can be held overnight if a thermal cycler or thermomixer with a heated lid is used to prevent evaporation inside the tube.

5.5 Incubate at  Room temperature for  00:10:00 .

10m



Note

DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to  Room temperature and thoroughly mixed before use. The DNA Purification Beads are a user-supplied reagent and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC+ kit.

5.6 Add  225 μ L of DNA Purification Beads, mix thoroughly, and incubate at  Room temperature for  00:05:00 .

5m



5.7 Place sample against magnet, and incubate until solution is clear.



5.8 Discard supernatant. While sample is still against magnet, add $\text{500 } \mu\text{L}$ of freshly prepared 80% ethanol, and incubate at Room temperature for $00:01:00$.

1m



5.9 Discard supernatant. While sample is still against magnet, add $\text{500 } \mu\text{L}$ of 80% ethanol, and incubate at Room temperature for $00:01:00$.

1m



5.10 Discard supernatant. While sample is still against magnet, incubate beads at Room temperature for 3-5 minutes to air-dry the beads.



5.11 Remove sample from magnet, resuspend beads thoroughly in $\text{50 } \mu\text{L}$ of Elution Buffer, and incubate at Room temperature for $00:05:00$.

5m



5.12 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.



5.13 Quantify sample using a Qubit following manufacturers protocol. Use $\text{2 } \mu\text{L}$ of DNA for the Qubit assay. The total DNA yield corresponds to the amount of DNA obtained from 0.5 million mammalian cells.



5.14 Estimate how many mammalian cells to use per Arima-HiChIP reaction. See the example below:

Example: We recommend aiming to use crosslinked material comprising $15 \mu\text{g}$ of DNA per Arima-HiChIP reaction. If an average of $2 \mu\text{g}$ of DNA was obtained from 10 mg of original input material as calculated in Step 13 then the sample yields 200 ng of DNA per mg of input tissue. This value is highly variable between different tissue types due to the density of cells in the tissue and the how difficult the tissue is to dissociate. To achieve an input of 0 ng , for this example, 75 mg of the original tissue mass must be input into each reaction. If 200 mg of tissue total were processed during the dissociation then $0.375x$ of the processed material must be added to one reaction. Additionally, please note that the crosslinked cell pellet for one Arima-HiChIP reaction should occupy no more than $20 \mu\text{L}$ of volume in the sample tube. If the crosslinked cell pellet comprises $15 \mu\text{g}$ of DNA but occupies greater than $20 \mu\text{L}$ of volume, aliquot the cells into multiple Arima-

HiChIP reactions such that the sum of the DNA input from all reactions is $\text{15 } \mu\text{g}$ and each cell pellet occupies no more than $\text{20 } \mu\text{L}$ of volume, or contact Technical Support for additional guidance.

HiChIP Protocol

3h 40m

- 6 **Input:** Crosslinked cells containing $\text{15 } \mu\text{g}$ of DNA

Output: Immunoprecipitated proximally-ligated DNA fragments

Overview: Arima-HiChIP begins with generating biotin-labelled proximally-ligated chromatin using Arima-HiC chemistry (Sections 1-3). The proximally-ligated chromatin is then sheared using Covaris (Section 8) or Diagenode Bioruptor (Section 9) instruments and bound to an antibody overnight. In parallel, Protein A beads are blocked overnight (**Section 3.3**) to prepare for immunoprecipitation. On the following day, the antibody-bound proximally-ligated chromatin is immunoprecipitated on Protein A beads, reverse crosslinked, and purified (**Section 3.4**). The resulting proximally-ligated DNA may be subject to quality control analysis described in the Quality Control section, and is then converted to sequence-ready Arima-HiChIP libraries following a custom protocol in a separate Arima-HiChIP Library Preparation user guide.

6.1 Perform Arima-HiC

Before you begin: The pellet of previously crosslinked cells or nuclei for one Arima-HiChIP reaction should occupy no more than $\text{20 } \mu\text{L}$ of volume and should be devoid of any residual liquid. If the cell pellet occupies greater than $\text{20 } \mu\text{L}$ of volume, aliquot the cells such that the sum of the DNA input from all reactions is between $\text{10-15 } \mu\text{g}$ and each cell pellet occupies no more than $\text{20 } \mu\text{L}$ of volume, or contact Technical Support for additional guidance. Note that Steps 2 – 3 require consecutive heated incubations. Make sure your thermal device(s) are pre-set to 62°C and 37°C for these incubations to avoid prolonged waiting periods before and between heated incubations.

Note

Steps 7.9, 7.19, and 7.21 require addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix tables.

6.2 Aliquot cells for the Arima-HiChIP reactions by resuspending the crosslinked cell pellet from above in a total volume of 1 mL of 1x PBS Fisher Scientific Catalog #BP243820 .



Visually measure the volume of the cell pellet (50-100 µL) and subtract it from 1ml to determine the amount of cold 1x PBS to resuspend the cells in. For instance, if the cell pellet is 50 µL of volume, resuspend it in 950 µL of cold

1x PBS Fisher Scientific Catalog #BP243820 .

6.3 Aliquot the amount of cells required for 15 µg based on *Estimating Input Amount* (Sections 4-5) above by taking the proportion of the cell pellet that corresponds to the amount of cells determined to yield 15 µg . For instance, if the cell pellet has 10 million cells and the *Estimating Input Amount* calculated that 4 million cells is needed for each HiChIP reaction then remove 400 µL from the resuspended cell pellet and place in a new 1.5 mL tube .



Note

Some cell pellets settle easily; therefore, it is recommended to mix by pipetting 5 times with a 1ml pipette prior to aliquoting the cells. For dissociated tissue pellets, resuspend in a total volume of 1 mL of 1x PBS Fisher Scientific Catalog #BP243820 , subtracting the volume of the cell pellet. Make sure the sample is homogenous then remove the fraction of the material recommended from *Estimating Input Amount* step.

6.4 Pellet the cell aliquot and the remainder of the resuspended crosslinked cell pellet by centrifugation at 1.000 x g, 4°C, 00:05:00 .

5m



6.5 Remove the supernatant from the cell pellets.

6.6 Re-freeze the crosslinked cell pellet on dry ice or liquid nitrogen, and store at -80 °C .



6.7 Resuspend the aliquot of crosslinked cells prepared in step 3 above in 20 µL of Lysis Buffer in a tube or a well of a PCR plate, and incubate at 4 °C for 00:20:00 .

20m



6.8 Add $\text{24 } \mu\text{L}$ of Conditioning Solution, mix gently by pipetting, and incubate at 62°C for $00:10:00$. If using a thermal cycler, set the lid temperature to 85°C .

10m



6.9 Add $\text{20 } \mu\text{L}$ of Stop Solution 2, mix gently by pipetting, and incubate at 37°C for $00:15:00$. If using a thermal cycler, set the lid temperature to 85°C .

15m



6.10 Add $\text{12 } \mu\text{L}$ of a master mix containing the following reagents:



| Reagent | Volume per reaction | $\times 10\%$ extra | $\times \#$ of reactions | Final |
|-------------|---------------------|---------------------|--------------------------|-------------------|
| • Buffer A | $7\mu\text{L}$ | $7.7\mu\text{L}$ | 2 | $15.4\mu\text{L}$ |
| • Enzyme A1 | $1\mu\text{L}$ | $1.1\mu\text{L}$ | 2 | $2.2\mu\text{L}$ |
| • Enzyme A2 | $4\mu\text{L}$ | $4.4\mu\text{L}$ | 2 | $8.8\mu\text{L}$ |
| Total | $12\mu\text{L}$ | | | $26.4\mu\text{L}$ |

Note

If sonication is to be performed on the same day as HiC, please prepare R2 Buffer (Appendix B), during the $01:00:00$ incubation in the step below. If shearing chromatin using a Covaris instrument, also prepare CS Buffer and MR1 Buffer (Appendix C). If shearing chromatin using a Diagenode Bioruptor Pico instrument prepare R1 Buffer (Appendix D). Preview sub-sections 3.2 and 3.3 below prior to execution.

6.11 Mix gently by pipetting, and incubate at 37°C for $01:00:00$. If using a thermal cycler, set the lid temperature to 85°C .

1h



6.12 Transfer sample to a clean 1.5 mL microfuge tube.

6.13 Pellet sample by centrifugation at $10.000 \times g$, 4°C , $00:10:00$.

10m



6.14 Carefully discard supernatant, without disturbing the sample pellet.

6.15 Gently add  1.5 mL Deionized Water, without disturbing the sample pellet.



6.16 Pellet sample by centrifugation at  10.000 x g, 4°C, 00:10:00 .

10m



6.17 Carefully discard supernatant, without disturbing the sample pellet.

6.18 Resuspended sample pellet in  75 µL Deionized Water by gently pipetting 5-10 times.



6.19 Transfer sample to a clean  1.5 mL microfuge tube , PCR tube, or PCR plate.



6.20 Add  16 µL of a master mix containing the following reagents:



| Reagent | Volume per reaction | x 10% extra | x # of reactions | Final |
|------------|---------------------|-------------|------------------|--------|
| • Buffer B | 12µL | 13.2µL | 2 | 26.4µL |
| • Enzyme B | 4µL | 4.4µL | 2 | 8.8µL |
| Total | 16µL | | | 35.2µL |

6.21 Mix gently by pipetting, and incubate at  Room temperature for  00:45:00 .

45m



6.22 Add  82 µL of a master mix containing the following reagents:



| Reagent | Volume per reaction | x 10% extra | # reactions | E | Final |
|------------|---------------------|-------------|-------------|---|---------|
| • Buffer C | 70µL | 77µL | 2 | | 154µL |
| • Enzyme C | 12µL | 13.2µL | 2 | | 26.4µL |
| Total | 82µL | | | | 180.4µL |

6.23 Mix gently by pipetting, and incubate at Room temperature for 00:15:00 . 15m



6.24 Store samples at -20 °C for up to 3 nights before proceeding with Chromatin Shearing.



Sample should be stored as-is. There is no need to remove supernatant at this time. After sample is removed from -20 °C storage, proceed with step 24.

6.25 If shearing settings are to be optimized using the instructions in Appendix F, it is recommended to store test samples and proceed with shearing optimization on the following day.

6.26 If, the user intends to optimize shearing settings for specific samples, follow the instructions in Appendix F before proceeding with step 26. If shearing will be performed with the recommended settings provided by Arima, or with settings previously optimized for the sample type/sonicator combination being used then please proceed with step 26.

6.27 Mix gently by inversion, and then immediately transfer 10 µL of sample into a new tube labelled "Ligation QC". Store the Ligation QC sample at -20 °C until later use in a following *Quality Control* section, and proceed to the next step with the remaining sample.

6.28 If sample is in a PCR tube or PCR plate, transfer sample to a clean 1.5 mL microfuge tube .

6.29 Pellet sample by centrifugation at 10.000 x g, 4°C, 00:10:00 . 10m



6.30 Discard supernatant. Once completed, store sample on On ice and immediately proceed to the appropriate *Chromatin Shearing and Antibody Binding sections* depending on use of a Covaris (Section 7) or Diagenode Bioruptor Pico (Section 8) instrument.

7 Chromatin Shearing and Antibody Binding (Covaris Workflow)

Before you begin: We have extensively validated the Covaris S220 instrument for chromatin shearing within the Arima-HiChIP Protocol, using the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tubes (Covaris SKU: 520045). Other Covaris instruments have also been used successfully. Please do not attempt to substitute any reagents or change any steps as this may result in a decrease in chromatin shearing efficiency and reproducibility.

7.1 Add 130 µL of cold CS Buffer to sample pellet, and resuspend by gentle pipette mixing.



7.2 Transfer 130 µL of sample to a Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube

7.3 Shear each sample using the Covaris instrument. Always store samples on On ice except when the sample is being sheared.

7.4 If shearing was optimized using the instructions in Appendix F, apply the optimized settings determined for each specific sample type.

7.5 If recommended shearing settings are to be used, exemplary shearing parameters using a Covaris S220 instrument on 2% crosslinked cells are provided below. If using a different Covaris instrument, ensure that the total energy delivered is the same as the below recommendations. Please contact Technical Support for any questions regarding chromatin shearing or for additional guidance.

| Setting | 2% Crosslinked Cells |
|---------------------------|----------------------|
| Setting | Value |
| Setpoint Temperature (°C) | 4 |
| Min/Max Temperature (°C) | 3-6 |
| Peak Incident Power (W) | 105 |
| Duty Factor (%) | 5 |
| Cycles per Burst | 200 |
| Treatment time (sec) | 300 |

Example Shearing Parameters Covaris S220

7.6 Transfer sheared sample to a 1.5 mL tube. Gently flick or spin the Covaris microTUBE to ensure all sample has been collected and transferred.



Note

After collecting the Shearing QC aliquot in the step below, we recommend starting the Reverse Crosslinking and DNA Purification protocol on the Ligation QC and Shearing QC aliquots as described in the *Quality Control* section (Section 4.1) and letting the reverse crosslinking reaction incubate overnight. The remainder of the Reverse Crosslinking and DNA Purification protocol will be completed during the 4hr Chromatin Immunoprecipitation reaction (Section 3.4) on the following day. This maximizes workflow efficiency and enables completion of the Arima-HiChIP Protocol and Quality Control sections within 2 days.

- 7.7 Transfer  10 µL of sheared sample into a new tube labelled "Shearing QC". Store the Shearing QC sample at  -20 °C until later use in the *Quality Control* section, and proceed to the next step with the remaining sample.  
- 7.8 Preclear the chromatin by adding  30 µL of Protein A Beads to a new  1.5 mL microfuge tube for each sample. 
- 7.9 Place Protein A Beads against magnet, and incubate until solution is clear. 
- 7.10 Discard supernatant.
- 7.11 Remove Protein A Beads from magnet, resuspend Protein A Beads in  880 µL of cold MR1 Buffer, and mix gently by pipetting. Do not vortex.  
- 7.12 Add the remaining  120 µL of sheared sample from step 7.6, to bring the total volume to  1 mL. Mix gently by pipetting. Do not vortex.  
- 7.13 Incubate for >1hr at  4 °C on a nutator, rotator, orbital shaker or equivalent device. 
- 7.14 Sample should rotate at  4 °C while completing *Reverse Crosslinking and DNA Purification* in section 9 of the protocol.

Note

If completion of the *Reverse Crosslinking and DNA Purification* in section 9 takes less than 1 hour, ensure samples rotate for at least 1 hour to ensure sufficient pre-clearing.

8 Chromatin Shearing and Antibody Binding – Diagenode Bioruptor Pico Workflow

Before you begin: We have validated the Diagenode Bioruptor Pico instrument for chromatin shearing within the Arima-HiChIP Protocol. Note that the efficiency of chromatin shearing on the Diagenode Bioruptor Pico is very sensitive to the sample shearing volume. The total sample volume after the completion of Step 8.3 should be exactly as indicated.

- 8.1 Add  110 µL of cold R1 Buffer to sample pellet, and resuspend by gentle pipette mixing. 
- 8.2 Incubate at  4 °C for  00:20:00.  20m
- 8.3 Transfer exactly  110 µL of sample to a Diagenode  0.65 mL microtube. 
- 8.4 Shear sample using the Diagenode Bioruptor Pico instrument

Note

If shearing was optimized using the instructions in Appendix F, apply the optimized settings for each sample type in the experiment. Vortex and quick spin the sample in a microfuge every 3 cycles to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing. Vortexing and quickly spinning the sample is critical for good shearing profiles and reproducibility between samples.

Note

If recommended shearing settings are to be used, use 30" ON / 30" OFF cycling conditions and 20 cycles total. Vortex and quick spin the sample in a microfuge every 3 cycles to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing. Note: Vortexing and quickly spinning the sample is critical for good shearing profiles and reproducibility between samples.



- 8.5 Transfer sheared sample to a 1.5 mL microfuge tube . Gently flick or spin the Diagenode 0.65 mL microtube to ensure all sample has been collected and transferred.

Note

After collecting the Shearing QC aliquot in the step below, we recommend starting the Reverse Crosslinking and DNA Purification (Section 4.1) protocol on the Ligation QC and Shearing QC aliquots as described in the Quality Control section and letting the reverse crosslinking reaction incubate overnight. The remainder of the Reverse Crosslinking and DNA Purification protocol will be completed during the 4hr Chromatin Immunoprecipitation reaction (Section 3.4) on the following day. This maximizes workflow efficiency and enables completion of the Arima-HiChIP Protocol and Quality Control within 2 days.

- 8.6 Transfer 10 μL of sheared sample into a new tube labelled "Shearing QC". Store the Shearing QC sample at -20 $^{\circ}\text{C}$ until use in the *Quality Control* section, and proceed to the next step with the remaining sample.
- 8.7 Preclear the chromatin by adding 30 μL of Protein A Beads to a new 1.5 mL microfuge tube for each sample.
- 8.8 Place Protein A Beads against magnet, and incubate until solution is clear.
- 8.9 Discard supernatant.
- 8.10 Remove Protein A Beads from magnet, resuspend Protein A Beads in 900 μL of cold R1 Buffer, and mix gently by pipetting. Do not vortex.
- 8.11 Add to the remaining 100 μL of sheared sample from step 8.5 above, to bring the total volume to 1 mL . Mix gently by pipetting. Do not vortex.
- 8.12 Incubate for >1 hr at 4 $^{\circ}\text{C}$ on a nutator, rotator, orbital shaker or equivalent device.

- 8.13 Sample should rotate at  4 °C while completing the *Reverse Crosslinking and DNA Purification* in section 9 of the protocol.

Note

If completion of the *Reverse Crosslinking and DNA Purification* in section 9 takes less than 1 hour, ensure samples rotate for 1 hour to ensure sufficient pre-clearing.

9 Reverse Crosslinking and DNA Purification

Overview: In this section, chromatin from the Ligation QC and Shearing QC aliquots collected during the Arima-HiChIP Protocol is reverse crosslinked and purified (Aliquots were collected in steps 7.6 and 8.5). The Shearing yield will be used to adjust the amount of Antibody added during the Immunoprecipitation to ensure high specificity capture of epitopes in the immunoprecipitation.

Before you begin: This sub-section describes the reverse crosslinking and purification of DNA from the Ligation QC and Shearing QC aliquots collected during the Arima-HiChIP Protocol. The DNA purified during this sub-section will be used in sub-sections 4.1 and 4.2 that follow. This protocol can be performed in microfuge tubes, PCR tubes, or PCR plates.

Note

Step 3 requires addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix tables.

- 9.1 Thaw all Ligation QC and Shearing QC aliquots.

- 9.2 Add  90 µL Elution Buffer to each Ligation and Shearing QC aliquot, to bring the total volume to  100 µL .



Note

Enzyme D should be warmed to RT to prevent precipitation in the below master mix. The remainder of this protocol is applied to both sets of QC aliquots.

9.3 Add  20.3 µL of a master mix containing the following reagents:



| Reagent | Volume per reaction | x 10% extra | x # of reactions | Final |
|------------|---------------------|-------------|------------------|--------|
| • Buffer D | 6µL | 6.6µL | 2 | 13.2µL |
| • Enzyme D | 14.3µL | 15.7µL | 2 | 31.4µL |
| Total | 20.3µL | | | 44.6µL |

9.4 Add  11.4 µL of Buffer E, mix gently by pipetting, and incubate as follows. If using a thermal cycler, set the lid temperature to  85 °C .



| Temperature | Time |
|-------------|-----------|
| 55 deg C | 30 min. |
| 68 deg C | 90 min.* |
| 25 deg C** | 10 min.** |

Note

*Do not incubate at  68 °C for longer than  01:30:00 unless doing so using a thermal cycler or thermomixer with a heated lid.

** To provide flexibility, this incubation can also be held overnight at  4 °C , in which case, the sample may turn slightly opaque or have precipitation. Warm sample to room temperature to re-dissolve the precipitate before proceeding to purification.

Note

DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to RT and thoroughly mixed before use. The DNA Purification Beads are a user-supplied reagent and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC+ kit.

- 9.5 Add $\text{120 } \mu\text{L}$ of DNA Purification Beads, mix thoroughly, and incubate at

Room temperature for $00:05:00$.

5m



- 9.6 Place sample against magnet, and incubate until solution is clear.



- 9.7 Discard supernatant. While sample is still against magnet, add $\text{200 } \mu\text{L}$ of freshly prepared

80% ethanol, and incubate at Room temperature for $00:01:00$.

1m



- 9.8 Discard supernatant. While sample is still against magnet, add $\text{200 } \mu\text{L}$ of 80% ethanol, and incubate at Room temperature for $00:01:00$.

1m



- 9.9 Discard supernatant. While sample is still against magnet, incubate beads at

Room temperature for 3-5 min. to air-dry the beads.



- 9.10 Remove sample from magnet, resuspend beads thoroughly in $\text{30 } \mu\text{L}$ of Elution Buffer, and incubate at Room temperature for $00:05:00$.

5m



- 9.11 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.



- 9.12 Quantify sample using a Qubit. Use $\text{2 } \mu\text{L}$ of DNA for the Qubit assay.



- 9.13 Record the sample concentration of the Shearing QC samples in the Arima-HiChIP QC Worksheet on the ChIP Efficiency tab under "Shearing QC Samples". Use the worksheet to calculate the ChIP Efficiency value. Store remaining sample at $\text{-20 } ^\circ\text{C}$ until use in the *Chromatin Fragmentation QC* section below (Section 4.2).



Note

Record the sample concentration of the Ligation QC samples in the Arima-HiChIP QC Worksheet on the Arima-QC1 tab under "Ligation QC Samples". Use the worksheet to calculate how much sample to use as input to the Arima-QC1 sub-section below (Section 4.3) and store remaining sample at  -20 °C until use in the *Chromatin Fragmentation QC* and *Arima-QC1* sections.

10 Antibody Addition



Overview: The success of an Arima HiChIP library is highly dependent on the success of the antibody binding step. This can vary depending on the type of antibody and available genome target locations. Too much antibody will bind non-specific targets and result in poor library specificity. To little antibody can result in poor library complexity. The steps below provide guidance for how to calculate the optimal mass of antibody to add to each sample to ensure the best chance of a successful HiChIP reaction.

Before you begin: This sub-section describes how to calculate the optimal mass of antibody to add to an individual reaction based on shearing yield, which is needed before proceeding with the Antibody binding step. The calculation is based on the following formula:

$$\text{Mass of Antibody} = \text{Shearing Yield} \times \text{Antibody Ratio}$$

- 10.1 Remove pre-clearing samples from  4 °C (step 7.14 or 8.13), and place against magnet, and incubate until solution is clear.



- 10.2 Transfer the supernatant (precleared chromatin) to a new  1.5 mL microfuge tube .



Note

The step below specifies the addition of antibody. If the concentration of the antibody is not provided by the vendor (e.g. H3K4me3 antibody (Millipore Cat # 04-745)), calculations can be based on a concentration of 1L/1g.

- 10.3 Use the worksheet in the Arima-HiChIP QC Worksheet on the ChIP Efficiency tab under "Shearing QC Samples"



- 10.4 Add antibody as calculated in column H, “ug of Antibody to add to sample”, and mix the entire sample gently by pipetting. Do not vortex.
- 10.5 The ratio for each antibody is listed in the Table below. This is calculated automatically in the Arima HiChIP QC Worksheet on the ChIP Efficiency tab under “Shearing QC Samples”, column H, by multiplying the g of shearing yield by the ratio listed in the table on the next page.

| A | B | C | D |
|----------|--------------|----------------|--------------------------------|
| Antibody | Manufacturer | Catalog# | Antibody:Shearing Yield Ratio* |
| H3K27ac | Active Motif | 91193 or 91194 | 0.2 |
| H3K4me1 | Thermofisher | 710795 | 0.4 |
| H3K4me2 | Active Motif | 39079 or 39679 | 0.4 |
| H3K4me3 | Millipore | 04-745 | 0.4 |
| H3K79me2 | Millipore | 04-835 | 0.4 |
| CTCF | Active Motif | 91285 | 0.5 |
| POLII | Active Motif | 39097 or 39497 | 0.4 |
| Rad 21 | Abcam | ab992 | 0.5 |

Antibody Ratio Table

- 10.6 Incubate at 4 °C overnight on a nutator, rotator, orbital shaker or equivalent device.



- 10.7 Immediately after setting up the overnight incubation in the prior step, proceed to the Bead Blocking sub-section (Section 11) directly below.

11 Bead Blocking

Before you begin: This sub-section prepares the Protein A beads that will be used in the Chromatin Immunoprecipitation sub-section (Section 12) on the following day. To increase the specificity of the chromatin immunoprecipitation reaction, the Protein A beads are “blocked” overnight via incubation with R2 Buffer, which contains BSA.

- 11.1 Add 30 µL of Protein A Beads to a new 1.5 mL microfuge tube for each sample.



- 11.2 Place Protein A Beads against magnet, and incubate until solution is clear.



11.3 Discard supernatant.

11.4 Remove Protein A Beads from magnet, resuspend Protein A Beads in  500 µL R2 Buffer, and mix gently by pipetting. Do not vortex.



11.5 Incubate at  4 °C overnight on a nutator, rotator, orbital shaker or equivalent device.



12 Chromatin Immunoprecipitation (ChIP)

2h

Before you begin: The chromatin immunoprecipitation sub-section comprises conjugating the antibody-bound chromatin to blocked Protein A Beads, rigorous washing, reverse crosslinking and purification of the immunoprecipitated proximally-ligated DNA fragments. This section requires use of a thermomixer pre-cooled to  4 °C, which may take at least  02:00:00. Alternatively, the thermomixer can be placed in a cold room or refrigerator.

Note

Step 12.15 requires addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix tables.

12.1 Set a thermomixer to  4 °C or place in a cold room or refrigerator and allow to cool for at least  02:00:00 for the thermomixer to reach  4 °C.

2h



12.2 Remove blocked Protein A Beads from  4 °C incubation, place against magnet, and incubate until solution is clear.



12.3 Discard supernatant.

12.4 Remove Protein A Beads from magnet, add  1 mL antibody-bound chromatin to the blocked Protein A Beads, and mix by pipetting until homogeneous.



- 12.5 Incubate at  4 °C for  04:00:00 on a nutator, rotator, orbital shaker or equivalent device.

4h

**Note**

Note: At the beginning of the 4hr incubation in the step below, please prepare R1 Buffer, R3 Buffer, LC Buffer, and LTE Buffer (Appendix E) and keep on ice until use. This will allow sufficient time for buffers to cool before their use in Steps 12.7-11. Also during this 4hr incubation, we recommend completing the Reverse Crosslinking and DNA Purification protocol on the Ligation QC and Shearing QC aliquots (Section 4.1), Chromatin Fragmentation QC (Section 4.2) and Arima-QC1 (Section 4.3) protocols described in the *Quality Control* section. This maximizes workflow efficiency and enables completion of the *Arima-HiChIP Protocol* and *Quality Control* within 2 days.

- 12.6 Place sample against magnet, and incubate until solution is clear.



- 12.7 Discard supernatant. Resuspend sample in  1 mL R1 Buffer, mix thoroughly by pipetting, and incubate on a thermomixer at  4 °C for  00:03:00 . with 1000 rpm shaking.

3m



- 12.8 Repeat Steps 6-7 two times using R1 Buffer, for a total of 3 R1 Buffer washes.



- 12.9 Repeat Steps 6-7 two times using R3 Buffer.



- 12.10 Repeat Steps 6-7 one time using LC Buffer.



- 12.11 Repeat Steps 6-7 two times using LTE Buffer.



- 12.12 Place sample against magnet, and incubate until solution is clear.



- 12.13 Discard supernatant.

Note

In the following step, the bead-bound sample is resuspended in Elution Buffer, but this does NOT elute the chromatin off the beads. The chromatin remains bound to the beads and the entire resuspended bead-bound sample is carried into Step 12.15 for reverse crosslinking and subsequent DNA purification.

- 12.14 Resuspend sample in 174 µL Elution Buffer and transfer the resuspended sample to a LoBind 1.5 mL microfuge tube . The resuspended sample can also be transferred into a PCR tube or plate for the completion of Steps 12.15-12.16 below.

Note

Enzyme D should be warmed to Room temperature to prevent precipitation in the below master mix.

- 12.15 Add 35.5 µL of a master mix containing the following reagents:

| A | B | C | D | E | F |
|----------|---------------------|-----------|---|-------------|--------|
| Reagent | Volume per reaction | 10% extra | | # reactions | Final |
| Buffer D | 10.5µL | 11.55µL | x | 2 | 23.1µL |
| Enzyme D | 25µL | 27.5µL | x | 2 | 55µL |
| Total | 35.5µL | | | | 78.1µL |

- 12.16 Add 20 µL of Buffer E, mix gently by pipetting, and incubate as follows. If using a thermal cycler, set the lid temperature to 85 °C .



| Temperature | Time |
|-------------|-----------|
| 55 deg C | 30 min. |
| 68 deg C | 90 min.* |
| 25 deg C ** | 10 min.** |

Note

*Do not incubate at $\text{68 }^{\circ}\text{C}$ for longer than 01:30:00 unless doing so using a thermal cycler or thermomixer with a heated lid.

** To provide flexibility, this incubation can also be held overnight at $\text{4 }^{\circ}\text{C}$, in which case, the sample may turn slightly opaque.

- 12.17 If sample is in a PCR tube or PCR plate, transfer sample into a LoBind

 1.5 mL microfuge tube .



Note

DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to Room temperature and mixed before use. The DNA Purification Beads are a user-supplied reagent and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC+ kit. The Protein A beads used to enrich for chromatin interactions, are not removed from the sample prior to adding the DNA Purification Beads below. This is for the purpose of avoiding sample loss and for convenience to the user.

- 12.18 Add  230 μL of DNA Purification Beads, mix thoroughly, and incubate at

Room temperature for 00:05:00 .

5m



- 12.19 Place sample against magnet, and incubate until solution is clear.



- 12.20 Discard supernatant. While sample is still against magnet, add  700 μL of freshly prepared 80% ethanol, and incubate at Room temperature for 00:01:00 .

1m



- 12.21 Discard supernatant. While sample is still against magnet, add  700 μL of 80% ethanol, and incubate at Room temperature for 00:01:00 .

1m



- 12.22 Discard supernatant. While sample is still against magnet, incubate beads at Room temperature for 3 – 5 min. to air-dry the beads.



12.23 Remove sample from magnet, resuspend beads thoroughly in $\text{50 } \mu\text{L}$ of Elution Buffer, and incubate at Room temperature for $00:05:00$.

5m



12.24 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.



Note

The following step utilizes $\text{2 } \mu\text{L}$ of sample material to quantify the amount of immunoprecipitated DNA. When using this amount of sample material, the lower limit of DNA quantification on a Qubit equates to an original sample concentration of $0.05\text{ng}/\mu\text{L}$, or 2.5 ng of immunoprecipitated DNA. If the Qubit reading is below the limit of detection, there is less than 2.5 ng of immunoprecipitated DNA but we still recommend proceeding to library preparation, assuming other QC metrics pass (see *Quality Control* section below).

12.25 Quantify sample using a Qubit. Use $\text{2 } \mu\text{L}$ of DNA for the Qubit assay.



12.26 Record the sample concentration in the Arima-HiChIP QC Worksheet on the ChIP Efficiency tab under "ChIP QC Samples".

12.27 If $>100\text{ng}$ of DNA is available, transfer 10 ng of sheared sample into a new tube labelled "ChIP QC" and store at $-20\text{ }^\circ\text{C}$ until later use in the Quality Control section.



12.28 Store remaining sample at $-20\text{ }^\circ\text{C}$ until ready to proceed to library preparation following an accompanying *Arima-HiChIP Library Preparation* user guide using Swift Biosciences Accel-NGS 2S Plus DNA Library Kit (Cat # 21024 or 21096).



Quality Control

13 **Overview:** In this section, multiple quality control analyses are performed to assess the efficiency of Arima-HiC, chromatin shearing, and chromatin immunoprecipitation. In sub-section 4.1, use the **Arima-HiChIP QC Worksheet** on the *Arima-QC1* tab under "Ligation QC

Samples" to calculate how much volume from the "Ligation QC" aliquot to use as input to the Arima-QC1 sub-section below (Section 4.2) and store remaining sample at -20°C until use in the *Chromatin Fragmentation QC* section. The DNA size from the proximally-ligated and sheared chromatin is analyzed to determine the efficiency of Arima-HiC and chromatin shearing. Also if available, the DNA size of the immunoprecipitated DNA is analyzed to confirm the expected size range of the immunoprecipitated DNA. Lastly in sub-section 4.2, the fraction of proximally-ligated DNA that has been labelled with biotin is analyzed using DNA purified from the Ligation QC aliquot. All these QC data are recorded and analyzed in the accompanying **Arima-HiChIP QC Worksheet**.

14 Chromatin Fragmentation QC

Before you begin: In this section, gel electrophoresis analysis will be used to evaluate the DNA size in the proximally-ligated and sheared chromatin, which determines the efficiency of Arima-HiC and chromatin shearing. This section will use the Ligation QC and Shearing QC aliquots purified in the previous section. If the ChIP QC aliquot has been collected during the *Arima-HiChIP Protocol*, this section can also be used to confirm the expected size range of the immunoprecipitated DNA. If performing this section concurrently with the Chromatin Immunoprecipitation protocol (Section 3.4), the ChIP QC aliquot will not be available yet.

- 14.1 Thaw the Ligation QC and Shearing QC aliquots. Thaw the ChIP QC aliquot if it has already been collected.
- 14.2 Analyze the DNA size of the proximally-ligated and sheared chromatin from the Ligation and Shearing QC samples, respectively, and the ChIP QC sample if one is available. Use gel electrophoresis systems such as a Bioanalyzer, TapeStation, or FlashGel. Exemplary results from the FlashGel system are below.

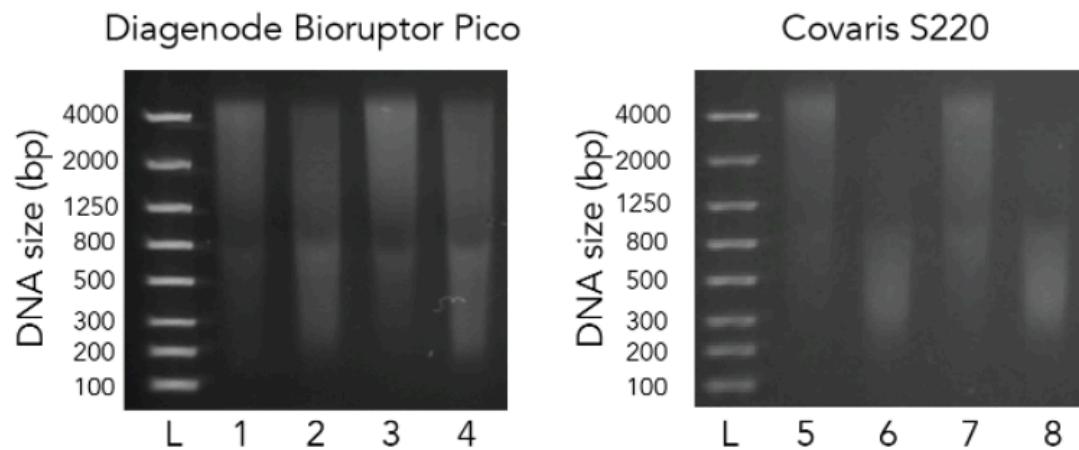


Fig.1 Expected DNA size distributions for proximally-ligated and sheared chromatin. Lonza 1.2% FlashGel electrophoresis analysis of DNA purified from proximally-ligated chromatin (Samples 1, 3, 5, and 7) and DNA purified after shearing using the Diagenode Bioruptor Pico instrument (Samples 2 and 4) and the Covaris S220 instrument (Samples 6 and 8).

15 Arima-QC1

Before you begin: The following protocol quantifies the fraction of proximally-ligated DNA that has been labelled with biotin, and is a quality control metric after completing the *Arima-HiChIP Protocol* but before proceeding to library preparation. The *Arima-QC1* protocol below uses QC Beads to enrich an aliquot of purified proximally-ligated DNA purified from the Ligation QC aliquot, which is quantified using a Qubit[®] fluorometer. Unlike standard Qubit[®] readings which involve quantifying a transparent unobstructed DNA sample, the Arima-QC1 value is obtained by quantifying DNA that is still bound to the QC Beads. This protocol can be performed in either plates or tubes. Set your thermal device (thermal cycler or thermomixer) to hold at 55°C. After completing the *Arima-QC1* protocol, use the provided *Arima-HiChIP QC Worksheet* to determine the Arima-QC1 values.

- 15.1 If necessary, thaw the Ligation QC samples prepared during Step 14 of the *Reverse Crosslinking and DNA Purification* protocol (Section 4.1) and transfer 75 ng of sample into a new tube labelled “Arima-QC1”.

- 15.2 Add Elution Buffer to bring the volume to 50 µL. The “Arima-QC1” sample should now contain 75 ng of proximally-ligated DNA in 50 µL of Elution Buffer.

- 15.3 Add $50 \mu\text{L}$ of QC Beads, mix thoroughly by pipetting, and incubate at
Room temperature for 00:15:00.
- 15.4 Place sample against magnet, and incubate until solution is clear.
- 15.5 Discard supernatant, and remove sample from magnet.
- 15.6 Wash beads by resuspending in 200 μL of Wash Buffer, and incubate at 55°C for 2 min.
- 15.7 Place sample against magnet, and incubate until solution is clear.
- 15.8 Discard supernatant, and remove sample from magnet.
- 15.9 Wash beads by resuspending in 200 μL of Wash Buffer, and incubate at 55°C for 2 min.
- 15.10 Place sample against magnet, and incubate until solution is clear.
- 15.11 Discard supernatant, and remove sample from magnet.
- 15.12 Wash beads by resuspending in 100 μL of Elution Buffer.
- 15.13 Place sample against magnet, and incubate until solution is clear.
- 15.14 Discard supernatant, and remove sample from magnet.
- 15.15 Resuspend beads in 7 μL of Elution Buffer. Proceed to next step with resuspended beads.

Note

The following step involves the quantification of the bead-bound DNA using the Qubit dsDNA HS Assay Kit.

15.16 Quantify the total amount of bead-bound DNA using Qubit. Use 2 μ L of thoroughly mixed bead-bound DNA for the Qubit assay.

15.17 Determine the Arima-QC1 value by following the Arima-HiChIP QC Worksheet. High quality Arima-QC1 values are expected to be >15%. If the Arima-QC1 value did not obtain a 'PASS' status, please contact Technical Support for troubleshooting assistance.

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Appendix A - Tissue Dissociation User-Supplied Buffers

17 Tissue Lysis Buffer 1 (TLB1), for 8 samples

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 8.33mM | 250 μ L |
| NaCl | Sigma | S5150-1L | 5M | 8.33mM | 50 μ L |
| IGEPAL CO-630* | Sigma | 542334 | 10%* | 0.167% | 500 μ L |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 16.667% | 5ml |
| Deionized Water** | Fisher Scientific | LC267402 | | | 24.2ml |
| | | | | Total | 30mL |

Note

* Stock IGEPAL comes as a 100% stock solution and must be diluted to 10% prior to use.

** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

18 Tissue Lysis Buffer 2 (TLB2), for 8 samples

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 4.17mM | 125µL |
| NaCl | Sigma | S5150-1L | 5M | 4.17mM | 25µL |
| IGEPAL CO-630* | Sigma | 542334 | 10%* | 0.0.84% | 250µL |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 8.33% | 2.5ml |
| Deionized Water** | Fisher Scientific | LC267402 | | | 27.1ml |
| | | | | Total | 30mL |

Note

* Stock IGEPAL comes as a 100% stock solution and must be diluted to 10% prior to use.

** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

19 Sucrose Solution (SS), for 8 samples

| A | B | C | D | E | F |
|-------------------|--------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Sucrose | Sigma® | S5016-25G | 342.3g/mol | 1M | 10.27g |
| MgAc | Sigma® | 63052-100ML | 1M | 3mM | 90µL |
| Tris-HCl, pH 8.0 | Fisher Scientific® | 15-568-025 | 1M | 10mM | 300µL |
| Deionized Water** | Fisher Scientific® | LC267402 | | | 29.6ml |
| | | | | Total | 30mL |

Note

** UltraPure™ DNase/RNase-Free Distilled Water is an acceptable alternative.

Appendix B – Day 1 Universal User-Supplied Buffers

20 R2 Buffer

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 50µL |
| NaCl | Sigma | S5150-1L | 5M | 140mM | 140µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1mM | 10µL |
| Triton X-100* | Sigma | T8787-50ML | 10%* | 1% | 500µL |
| SDS | Fisher Scientific | MT-46040CI | 10% | 0.1% | 50µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.1% | 50µL |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 1% | 50µL |
| BSA | Fisher Scientific | AM2616 | 50mg/mL | 5mg/mL | 500µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 3.65mL |
| | | | | Total | 5mL |

Note

* Stock Triton X-100 comes as a 100% stock solution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the **R2 Buffer** formulation.

** Stock Triton X-100 comes as a 100% stock solution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the

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Appendix C – Day 1 Covaris® User-Supplied Buffers

22 CS Buffer

| A | B | C | D | E | F |
|------------------|--------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific® | 15-568-025 | 1M | 10mM | 50µL |
| SDS | Fisher Scientific® | MT-46040CI | 10% | 0.1% | 50µL |
| Deionized Water* | Fisher Scientific® | LC267402 | | | 4.9mL |
| | | | | Total | 5mL |

Note

* UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

23 MR1 Buffer

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 100µL |
| NaCl | Sigma | S5150-1L | 5M | 159mM | 319.2µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1.14mM | 22.8µL |
| Triton X-100* | Sigma | T8787-50ML | 10%* | 1.14% | 1.14mL |
| SDS | Fisher Scientific | MT-46040CI | 10% | 0.1% | 100µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.114% | 114µL |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 1.14% | 114µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 8.09mL |
| | | | | Total | 10mL |

Note

- * Stock Triton X-100 comes as a 100% stock solution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the **MR1 Buffer** formulation.
- ** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

Appendix D – Day 1 Bioruptor User-Supplied Buffers**24 R1 Buffer**

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 100µL |
| NaCl | Sigma | S5150-1L | 5M | 140mM | 280µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1mM | 20µL |
| Triton X-100* | Sigma | T8787-50ML | 10%* | 1% | 1mL |
| SDS | Fisher Scientific | MT-46040CI | 10% | 0.1% | 100µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.1% | 100µL |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 1% | 100µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 8.3mL |
| | | | | Total | 10mL |

Note

- * Stock Triton X-100 comes as a 100% stock solution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the **R1 Buffer** formulation.
- ** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

Appendix E – Day 2 Universal User-Supplied Buffers

25 R1 Buffer

| A | B | C | D | E | F |
|-----------------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 300µL |
| NaCl | Sigma | S5150-1L | 5M | 140mM | 840µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1mM | 60µL |
| Triton X-100* | Sigma | T8787-50ML | 10%* | 1% | 3mL |
| SDS | Fisher Scientific | MT-46040CI | 10% | 0.1% | 300µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.1% | 300µL |
| Protease Inhibitor Cocktail | Sigma | P8340-5ML | 100% | 1% | 300µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 24.9mL |
| | | | | Total | 30mL |

Note

* Stock Triton X-100 comes as a 100% stocksolution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the **R1 Buffer** formulation.

** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

26 R3 Buffer

| A | B | C | D | E | F |
|------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 200µL |
| NaCl | Sigma | S5150-1L | 5M | 300mM | 1.2mL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1mM | 40µL |
| Triton X-100* | Sigma | T8787-50ML | 10%* | 1% | 2mL |

| A | B | C | D | E | F |
|---------------------|-------------------|------------|-----|-------|---------|
| SDS | Fisher Scientific | MT-46040CI | 10% | 0.1% | 200µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.1% | 200µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 16.16mL |
| | | | | Total | 20mL |

Note

* Stock Triton X-100 comes as a 100% stock solution and must be diluted to 10% and mixed thoroughly until homogeneous prior to use in the **R3 Buffer** formulation.

** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

27 LC Buffer

| A | B | C | D | E | F |
|---------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 100µL |
| Lithium Chloride | Sigma | L7026-100ML | 8M | 150mM | 187.5µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 1mM | 20µL |
| IGEPAL CO-630* | Sigma | 542334 | 10%* | 0.5% | 500µL |
| Sodium Deoxycholate | Fisher Scientific | 50-255-884 | 10% | 0.1% | 100µL |
| Deionized Water** | Fisher Scientific | LC267402 | | | 9.093mL |
| | | | | Total | 10mL |

Note

* Stock IGEPAL comes as a 100% stock solution and must be diluted to 10% prior to use in the **LC Buffer** formulation.

** UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

- 28 **LTE Buffer** – The LTE Buffer is a *low EDTA* (0.1mM) TE Buffer that does not need to be prepared fresh directly before use in the *Arima-HiChIP Protocol*.

| A | B | C | D | E | F |
|------------------|-------------------|-------------|---------------------|---------------------|--------------|
| Reagent | Stock Vendor | Stock Cat # | Stock Concentration | Final Concentration | Stock Amount |
| Tris-HCl, pH 8.0 | Fisher Scientific | 15-568-025 | 1M | 10mM | 200µL |
| EDTA | Fisher Scientific | AM9260G | 0.5M | 0.1mM | 4µL |
| Deionized Water* | Fisher Scientific | LC267402 | | | 19.796mL |
| | | | | Total | 20mL |

Note

* UltraPure DNase/RNase-Free Distilled Water is an acceptable alternative.

Appendix F – Optional Shearing Titration

- 29 **Chromatin Shearing Optimization- Covaris Workflow**

Before you begin: The below steps are intended to be performed if one or more samples processed are intended for shearing optimization. Note: we recommend that shearing optimization be conducted for each sample type of interest and for each shearing platform being used. Following the below steps, the sample will likely be over-sheared and should not be included in subsequent steps.

- 29.1 Add 130µL of cold CS Buffer to sample pellet of the shearing optimization samples, and resuspend by gentle pipette mixing.
- 29.2 Transfer 130µL of sample to a Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube
- 29.3 Shear the sample following the steps below using a Covaris S220 instrument on 2% crosslinked cells. Always store samples on ice except when the sample is being sheared.

| A | B | C | D | E | F |
|----------------------------------|-----------------------------|----------|----------|----------|----------|
| Setting | 2% Crosslinked Cells | | | | |
| Total Time (minutes) | 1 | 3 | 4 | 7 | 9 |
| Setpoint Temperature (°C) | 4 | 4 | 4 | 4 | 4 |
| Min/Max Temperature (°C) | 3-6 | 3-6 | 3-6 | 3-6 | 3-6 |
| Peak Incident Power (W) | 105 | 105 | 105 | 105 | 105 |
| Duty Factor (%) | 5 | 5 | 5 | 5 | 5 |
| Cycles per Burst | 200 | 200 | 200 | 200 | 200 |
| Treatment time (sec) | 60 | 120 | 120 | 120 | 120 |

Table of recommended shearing settings for the Covaris S220

29.4 **1 minute:**

- Begin by sonicating the shearing optimization sample using the shearing parameters provided below under “1 minute”
- After completion of the 1 minute sonication, transfer 10uL of the sheared sample to a new tube labeled “1 minute”
- Add 10uL of CS buffer to the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

29.5 **3 minutes:**

- Sonicate the shearing optimization sample again using the shearing parameters provided below under “3 minutes”
- After completion of the 3 minute sonication, transfer 10uL of the sheared sample to a new tube labeled “3 minutes”
- Add 10uL of CS buffer to the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

29.6 **5 minutes:**

- Sonicate the shearing optimization sample again using the shearing parameters provided below under “5 minutes”

- b. After completion of the 5 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "5 minutes"
- c. Add 10uL of CS buffer to the Covaris microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

29.7 **7 minutes:**

- a. Sonicate the shearing optimization sample again using the shearing parameters provided below under "7 minutes"
- b. After completion of the 7 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "7 minutes"
- c. Add 10uL of CS buffer to the CovarismicroTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube, which contains the shearing optimization sample

29.8 **9 minutes:**

- a. Sonicate the shearing optimization sample again using the shearing parameters provided below under "9 minutes"
- b. After completion of the 9 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "9 minutes"
- c. Only 10uL is required for reverse crosslinking. The remaining 120uL can be discarded.

29.9 Please contact Technical Support for any questions regarding chromatin shearing or for additional guidance.

30 Reverse Crosslinking of Shearing Optimization Aliquots

Before you begin: This sub-section describes the reverse crosslinking and purification of DNA from the shearing optimization aliquots collected above. This protocol can be performed in microfuge tubes, PCR tubes, or PCR plates.

Note

Step 3 requires addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix table.

30.1 Thaw all shearing optimization aliquots.

30.2 Add 90 μ L **Elution Buffer** to each shearing optimization aliquot, to bring the total volume to 100 μ L.

Note

Enzyme D should be warmed to RT to prevent precipitation in the below master mix.

30.3 Add 20.3 μ L of a master mix containing the following reagent:

| A | B | C | D | E | F | G |
|--------------|------------------------------|--------------|---|-------------|---|---------------|
| Reagent | Volume per reaction | 10% extra | | # reactions | | Final |
| Buffer D | 6 μ L | 6.6 μ L | x | 5 | = | 33.0 μ L |
| Enzyme D | 14.3 μ L | 15.7 μ L | x | 5 | = | 78.5 μ L |
| Total | 20.3μL | | | | | 111.5 μ L |

30.4 Add 11.4 μ L of **Buffer E**, mix gently by pipetting, and incubate as follows. If using a thermal cycler, set the lid temperature to 85°C.

| A | B |
|-------------|-----------|
| Temperature | Time |
| 55°C | 30 min. |
| 68°C | 90 min.* |
| 25°C** | 10 min.** |

*Do not incubate at 68°C for longer than 90 min. unless doing so using a thermal cycler or thermomixer with a heated lid.

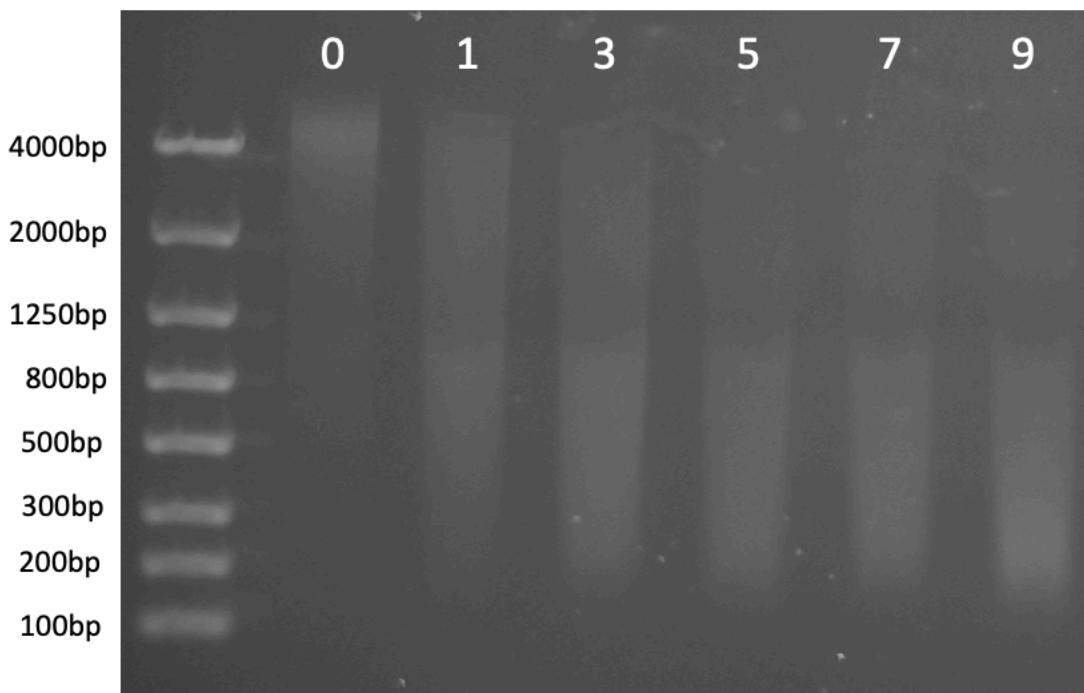
** To provide flexibility, this incubation can also be held overnight at 4°C, in which case, the sample may turn slightly opaque or have precipitation. Warm sample to room temperature to re-dissolve the precipitate before proceeding to purification.

Note

DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to RT and thoroughly mixed before use. The DNA Purification Beads are a *user-supplied reagent* and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC⁺ kit.

- 30.5 Add 120µL of DNA Purification Beads, mix thoroughly, and incubate at RT for 5 min.
- 30.6 Place sample against magnet, and incubate until solution is clear.
- 30.7 Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
- 30.8 Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
- 30.9 Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
- 30.10 Remove sample from magnet, resuspend beads thoroughly in 30µL of Elution Buffer, and incubate at RT for 5 min.
- 30.11 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
- 30.12 Analyze the DNA size of the sheared chromatin from the shearing optimization aliquots. 200-800bp. Use gel electrophoresis systems such as a Bioanalyzer, TapeStation, or FlashGelTM. Exemplary results from the FlashGel system are below.

Sonication Time (minutes)



FlashGel system example

31 Chromatin Shearing Optimization- Diagenode Bioruptor Pico Workflow

Before you begin: The below steps are intended to be performed if one or more samples processed are intended for shearing optimization. Note: we recommend that shearing optimization be conducted for each sample type of interest and for each shearing platform being used. Following the below steps, the sample will likely be over-sheared and should not be included in subsequent steps.

- 31.1 Add 110 μ L of cold R1 Buffer to sample pellet, and resuspend by gentle pipette mixing.
- 31.2 Incubate at 4°C for 20 min.
- 31.3 Transfer exactly 110 μ L of sample to a Diagenode 0.65mL Microtube.
- 31.4 **10 Cycles:**
 1. Shear sample using the Diagenode Bioruptor Pico instrument using 30" ON / 30" OFF cycling conditions and 10 cycles total.

2. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "10 cycles".
3. Add 10uL of **R1** buffer to the Diagenode 0.65mL Microtube which contains the shearing optimization sample.
4. Vortex and quick spin the sample in a microfuge to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing.

Note

Vortexing and quickly spinning the sample is *critical* for good shearing profiles and reproducibility between samples.

31.5 15 Cycles:

1. Shear sample using the Diagenode Bioruptor Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
2. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "15 cycles".
3. Add 10uL of **R1** buffer to the Diagenode 0.65mL Microtube which contains the shearing optimization sample.
4. Vortex and quick spin the sample in a microfuge to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing.

Note

Vortexing and quickly spinning the sample is *critical* for good shearing profiles and reproducibility between samples.

31.6 20 Cycles:

1. Shear sample using the Diagenode Bioruptor Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
2. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "20 cycles".
3. Add 10uL of **R1** buffer to the Diagenode 0.65mL Microtube which contains the shearing optimization sample.
4. Vortex and quick spin the sample in a microfuge to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing.

Note

Vortexing and quickly spinning the sample is *critical* for good shearing profiles and reproducibility between samples.

31.7 25 Cycles:

1. Shear sample using the Diagenode Bioruptor Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
2. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "25 cycles".
3. Add 10uL of **R1** buffer to the Diagenode 0.65mL Microtube which contains the shearing optimization sample Vortex and quick spin the sample in a microfuge to re-incorporate sample material that has been ejected out of solution onto the upper tube wall to provide more uniform and consistent shearing.

Note

Vortexing and quickly spinning the sample is *critical* for good shearing profiles and reproducibility between samples.

31.8 30 Cycles:

1. Shear sample using the Diagenode Bioruptor Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
2. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "30 cycles"
3. Only 10uL is required for reverse crosslinking. The remaining 120uL can be discarded.

32 Reverse Crosslinking of Shearing Optimization Aliquots**32.1** Thaw all shearing optimization aliquots.**32.2** Add 90 μ L Elution Buffer to each shearing optimization aliquot, to bring the total volume to 100 μ L.**Note**

Enzyme D should be warmed to RT to prevent precipitation in the below master mix.

32.3 Add 20.3 μ L of a master mix containing the following reagents:

| A | B | C | D | E | F | G |
|--------------|------------------------------|--------------|---|-------------|---|---------------|
| Reagent | Volume per reaction | 10% extra | | # reactions | | Final |
| Buffer D | 6 μ L | 6.6 μ L | x | 5 | = | 33.0 μ L |
| Enzyme D | 14.3 μ L | 15.7 μ L | x | 5 | = | 78.5 μ L |
| Total | 20.3μL | | | | | 111.5 μ L |

32.4 Add 11.4 μ L of **Buffer E**, mix gently by pipetting, and incubate as follows. If using a thermal cycler, set the lid temperature to 85°C.

| A | B |
|-------------|-----------|
| Temperature | Time |
| 55°C | 30 min. |
| 68°C | 90 min.* |
| 25°C** | 10 min.** |

*Do not incubate at 68°C for longer than 90 min. unless doing so using a thermal cycler or thermomixer with a heated lid.

** To provide flexibility, this incubation can also be held overnight at 4°C, in which case, the sample may turn slightly opaque or have precipitation. Warm sample to room temperature to re-dissolve the precipitate before proceeding to purification.

Note

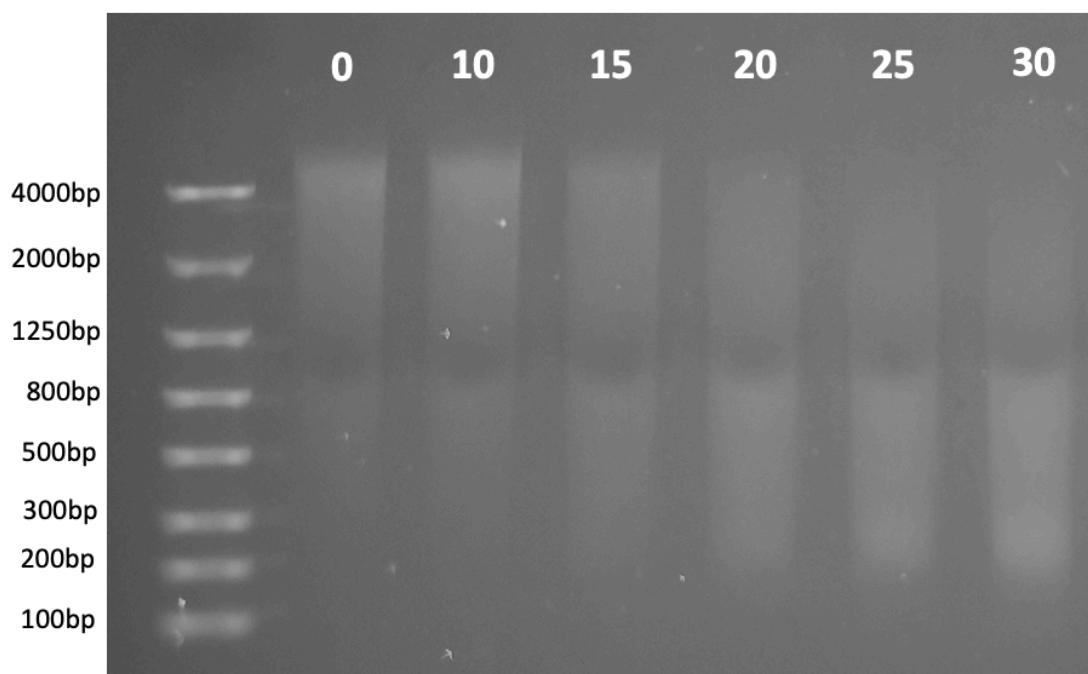
DNA Purification Beads (e.g. AMPure XP Beads) should be warmed to RT and thoroughly mixed before use. The DNA Purification Beads are a *user-supplied reagent* and should not be mistaken for the Enrichment Beads or QC Beads provided in the Arima-HiC⁺ kit.

32.5 Add 120 μ L of DNA Purification Beads, mix thoroughly, and incubate at RT for 5 min.

32.6 Place sample against magnet, and incubate until solution is clear.

- 32.7 Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
- 32.8 Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
- 32.9 Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
- 32.10 Remove sample from magnet, resuspend beads thoroughly in 30µL of Elution Buffer, and incubate at RT for 5 min.
- 32.11 Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
- 32.12 Analyze the DNA size of the sheared chromatin from the shearing optimization aliquots. The ideal size for sheared chromatin is 200-800bp. Use gel electrophoresis systems such as a Bioanalyzer, TapeStation, or FlashGel™. Exemplary results from the FlashGel system are below.

Sonication Time (cycles)



FlashGel system example results.

Protocol references

Fang, Rongxin, Miao Yu, Guoqiang Li, Sora Chee, Tristin Liu, Anthony D. Schmitt, and Bing Ren. "Mapping of Long-Range Chromatin Interactions by Proximity Ligation-Assisted ChIP-Seq." *Cell Research* 26, no. 12 (December 2016): 1345–48. <https://doi.org/10.1038/cr.2016.137>.

Acknowledgements

This HiChIP protocol, developed using Arima-HiC+ technology, is based on PLAC-seq (Proximity Ligation-Assisted ChIP-seq)¹. It combines *in situ* Hi-C and chromatin immunoprecipitation (ChIP) techniques to achieve targeted interrogation of chromatin organization at specific genomic regions.

The primary developers of the initial PLAC-seq are:

- Miao Yu
- Rongxin Fang

Additional acknowledgements to:

- Ivan Juric
- Armen Abnousi
- Ming Hu
- Bing Ren

(1) Fang et al., "Mapping of Long-Range Chromatin Interactions by Proximity Ligation-Assisted ChIP-Seq."