User Guide

Arima-HiC+ Kit

HiChIP User Guide for Mammalian Cells and Tissues using Transcription Factors and Histones
8 reactions

Material Part Number: A101020
Document Part Number: A160430 v02
Release Date: June 2021

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U.S. Patent No. US 9,434,985 and 9,708,648 pertains to the use of this product.

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Revision History

Document	Date	Description of Change
Material Part Number: A101020 **Document Part Number:** A160430 v00	December 2020	Initial Release
Material Part Number: A101020 **Document Part Number:** A160430 v01	March 2021	Included updated list of supported antibodies Included instructions for a safe stopping point following ligation of HiC material, prior to Chromatin Shearing Provided instructions for shearing optimization to be performed following the safe stopping point
Material Part Number: A101020 **Document Part Number:** A160430 v02	June 2021	Included updates for adding antibody based on the shearing QC aliquot yield

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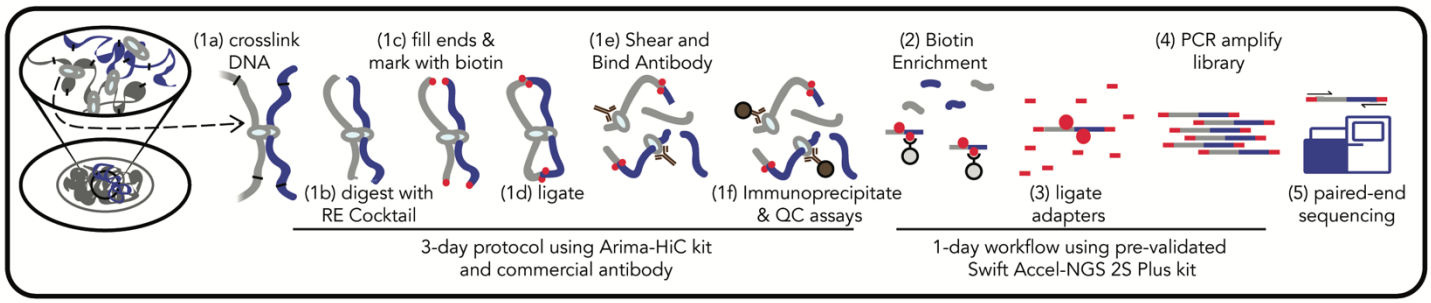


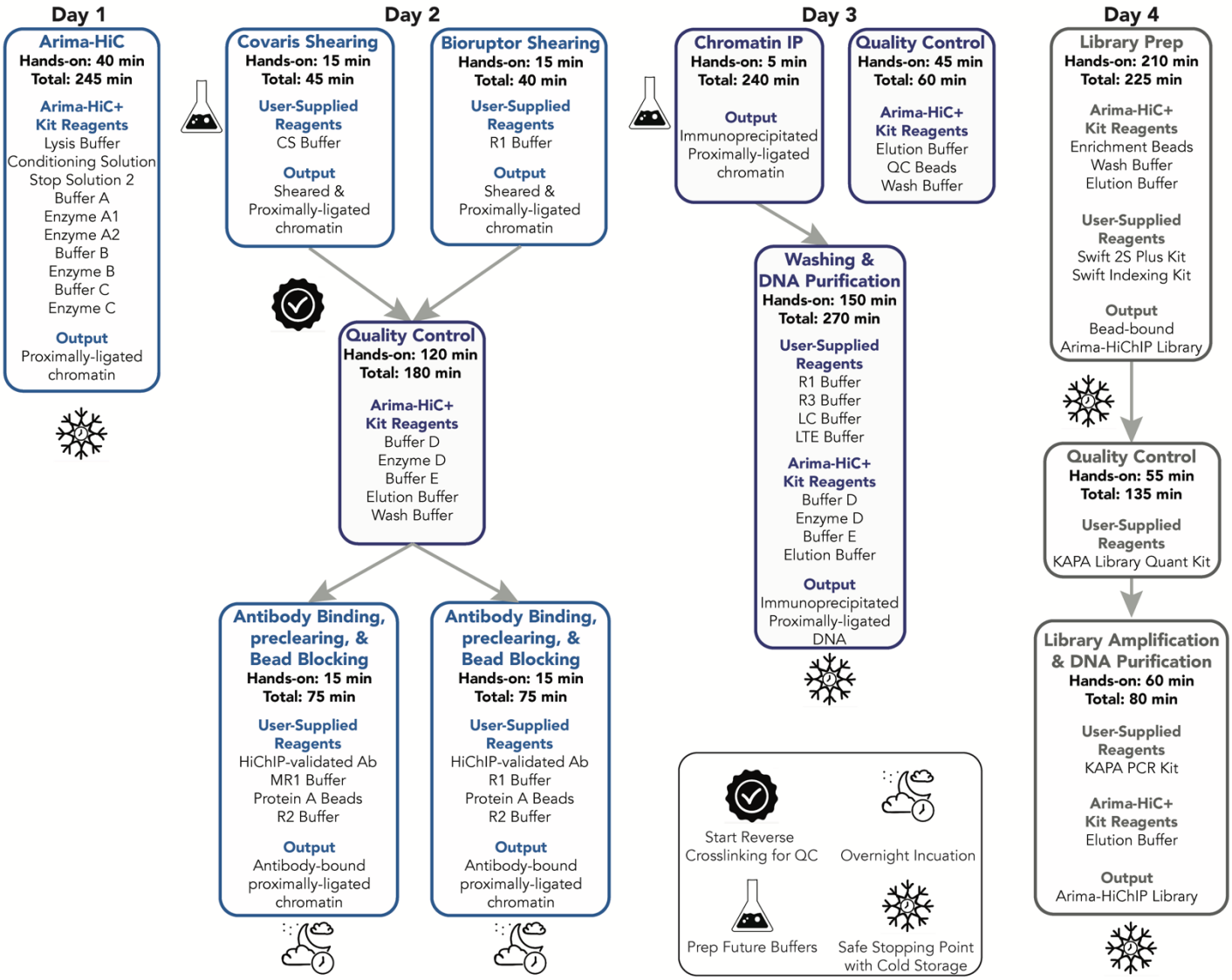
1.1. **Arima-HiChIP Workflow Overview**

Arima-HiChIP is an experimental workflow that captures the structure (three-dimensional conformation) of genomes associated with a protein of interest. As illustrated in the workflow schematic above, 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 Arima-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 Arima-HiChIP libraries.

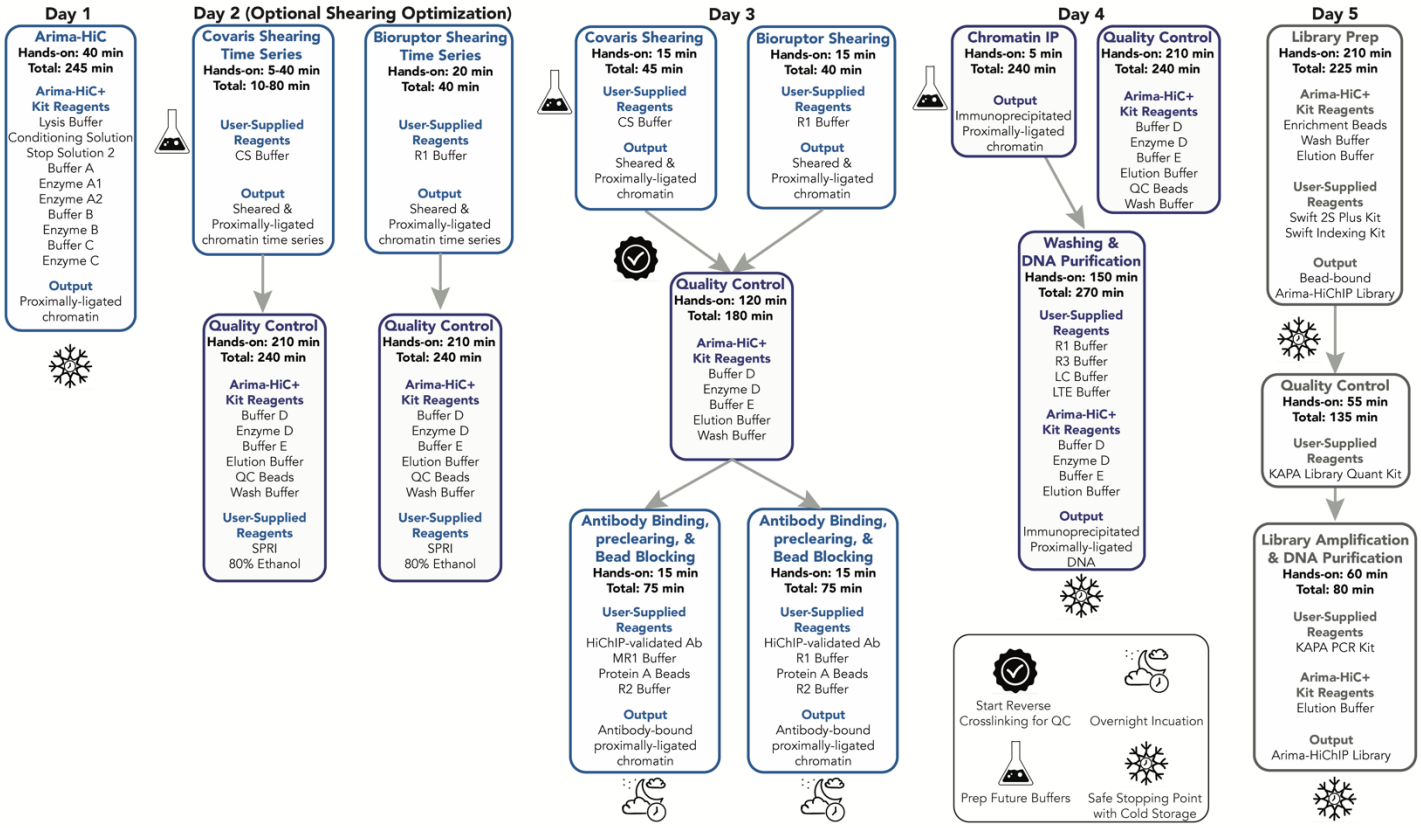
1.2. **Sequencing and Data Analysis**

Arima-HiChIP libraries are sequenced via Illumina® sequencers in "paired-end" mode. The resulting data is referred to as Arima-HiChIP data. Arima strongly recommends processing and analyzing Arima-HiChIP using the publicly available tools Feather and MAPS (**GitHub Link**; Juric, 2019). This recommendation resulted from internal benchmarking analyses of computational tools for HiChIP data analysis and a collaboration to optimize the outputs of MAPS for maximum compatibility with Arima-HiChIP. 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 Arima-HiChIP data, so please carefully review our Arima-HiChIP Bioinformatics User Guide or contact Technical Support for further assistance implementing these tools. Arima also recommends visualizing Arima-HiChIP data using the WashU Epigenome Browser (**http://epigenomegateway.wustl.edu**). For a more in-depth description of Arima-HiChIP data QC and analysis, please see Section 2.8, the Arima-HiChIP QC Worksheet, and the Arima-HiChIP Bioinformatics User Guide.

Arima-HiChIP 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.



Workflow option 2. Shearing Optimization. This workflow is best when the optimal shearing conditions for a given sample type and or sonication platform need to be determined on HiChIP material to optimize the immune precipitation.

2.1. Handling and Preparation

- The safe stopping point after the ligation step has been validated for up to 3 nights.
- Once sonication of the samples has started, there is no safe stopping point with cold storage until completing chromatin immunoprecipitation (ChIP) of the Arima-HiChIP Protocol. Please plan for at least two consecutive days of experimentation once sonication of samples has begun.
- If shearing conditions are unknown prior to beginning the HiChIP protocol, the user can process a sacrificial sample, along with other biological replicates of that sample type, and conduct a shearing time series of the sacrificial sample to determine the optimal shearing time and apply those conditions to the remaining biological replicates of that sample type that were processed in parallel. We recommend doing this for every cell type that has never been processed for HiChIP using the user's sonication platform of choice.
- Several steps during the *Arima-HiChIP Protocol* require preparation of a master mix. Sufficient reagent has been included in the kit to make master mixes with 10% excess volume. Use the master mix calculation tables provided.
- Throughout the protocol, room temperature (RT) is defined as 20 to 25°C.
- If any part of the *Arima-HiChIP Protocol* will be performed in PCR plates or PCR tubes, each tube or well must have a total volume capacity of at least 320µL and be compatible with thermal cyclers and other required equipment. See Section 2.2 for recommended PCR plates and PCR tubes. Use of seals and caps for PCR plates and tubes is required.
- All kit reagents should be fully thawed and thoroughly mixed before use.
- **Stop Solution 1**, ●**Conditioning Solution**, and ●**Buffer D** from **Box A** may contain precipitates. If present, these precipitates must be dissolved before use. Heating these reagents at 37°C for 5-15 minutes may be necessary to dissolve precipitates.
- During handling and preparation, reagents from **Box A** should be kept at RT.
- During handling and preparation, reagents from **Box B** should be kept on ice, except for **Enzyme D,** which should be kept on ice but warmed to room temperature just before use.
- Enzyme solutions from **Box B** are viscous and require special attention during pipetting.
- Following the HiChIP procedure, libraries are prepared for sequencing using the Swift Biosciences® Accel-NGS® 2S Plus DNA Library Kit (Cat # 21024 or 21096).

2.2. Universal user-supplied reagents, consumables, and equipment checklist

- 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, 40⌠m (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.)

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

- Validated 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)

2.4. User-Supplied Buffer Recipes

Execution of the *Arima-HiChIP Protocol* requires certain user-supplied buffers. The *Arima-HiChIP Protocol* section contains important instructions regarding when to prepare user-supplied buffers during the workflow, and the required buffers depend on which chromatin shearing instrument is used. All buffers, except LTE Buffer, should be prepared fresh on the day of experimentation. Once prepared, all buffers should be stored at 4°C until use. Recipes for these buffers are located in Appendices A-D, as described in the table below. Each recipe is sufficient for 8 Arima-HiChIP reactions and should be scaled accordingly if more or less than 8 reactions are processed simultaneously.

Tissue Dissociation (optional)		**Day 1 - Universal**		**Day 1 - Covaris® Only**		**Day 1 - Bioruptor® Only**		**Day 2 - Universal**	
Buffer Name	Recipe Location	Buffer Name	Recipe Location	Buffer Name	Recipe Location	Buffer Name	Recipe Location	Buffer Name	Recipe Location
TLB1 TLB2 SS	Appendix A	R2 Buffer	Appendix B	CS Buffer MR1 Buffer	Appendix C	R1 Buffer	Appendix D	R1 Buffer R3 Buffer LC Buffer LTE Buffer	Appendix E

2.5. Crosslinking

Chromatin crosslinking is a critical step of the *Arima-HiChIP Protocol*. We strongly recommend crosslinking using a final concentration of 2% formaldehyde for 10 minutes for Mammalian cell lines, pulverized soft tissue, and cryopreserved primary cells. We also recommend using formaldehyde that has been stabilized with methanol (see Section 2.2). These crosslinking conditions may appear "stronger" than some crosslinking protocols for conventional ChIP-seq, but have been observed to yield the highest quality Arima-HiChIP data. Crosslinking using a lower formaldehyde concentration, less time, or formaldehyde *not* stabilized with methanol may weaken the crosslinking and could result in reduced ChIP specificity. In turn, a less specific ChIP reaction will result in the need for more Raw Paired-End (PE) Reads to obtain reproducible loop calls from the Arima-HiChIP data. See Section 2.8 for further explanation regarding the relationship between ChIP specificity and chromatin loop discovery. On the other hand, over-crosslinking using a higher formaldehyde concentration or for more time could also result in sub-optimal data quality, due in part to challenges with Hi-C biochemistry and/or chromatin shearing.

2.6. Chromatin Shearing

Chromatin shearing is also a critical step of the *Arima-HiChIP Protocol*. It is recommended to regularly validate the expected performance of the shearing instrument and to adhere to the manufacturer's maintenance schedule for optimal shearing performance and experimental consistency. Please contact the shearing instrument's manufacturer technical support for any questions regarding chromatin shearing instruments to ensure optimal shearing performance. We provide shearing instrument settings designed to shear the proximally-ligated chromatin to an approximate size of 400bp, with the majority of DNA in the size range of ~200-800bp. Please note that sample-type specific shearing parameters that have previously been optimized

for conventional ChIP-seq are **not** expected to translate to optimal chromatin shearing parameters for Arima-HiChIP and present a significant risk of over-shearing the proximally-ligated chromatin leading to sub-optimal assay performance. Proximally-ligated chromatin is of significantly smaller molecular weight than the input chromatin for conventional ChIP-seq and has been subjected to a series of molecular biology reactions prior to chromatin shearing which also differs from conventional ChIP-seq. To optimize chromatin shearing conditions, the user has the option to include an additional replicate of each sample type to the experiment, to be consumed in a shearing time course and analyzed prior to shearing the remaining biological replicates using the optimized settings for that sample type. Please note, the shearing optimization sample will likely be over-sheared during this workflow and should not be included in subsequent steps. Alternatively, we have provided recommended chromatin shearing conditions for Covaris® or Diagenode® Bioruptor® Pico instruments that produce appropriate chromatin fragmentation for Arima-HiChIP.

2.7. Antibody Selection

Selection of an antibody with high specificity and sensitivity is also a critical factor in obtaining high quality Arima-HiChIP data. For example, a 2-fold reduction in ChIP specificity can double the number of Raw PE Reads required for chromatin loop discovery. Therefore, Arima has currently validated and recommends one H3K27ac antibody and one H3K4me3 antibody (see section 2.2) for highest quality Arima-HiChIP data. Using antibodies other than these may compromise the Arima-HiChIP data quality. We have observed widely variable lot-to-lot performance of polyclonal H3K27ac antibodies manufactured by the same vendor, and considerably less variability for our recommended monoclonal and recombinant antibodies (See Section 2.2). A set of ChIP peaks is needed for HiChIP analysis using the Arima-MAPS 2.0 Pipeline. The Arima-MAPS 2.0 ChIP pipeline will automatically call ChIP peaks from the HiChIP data using MACS2. The MACS2 parameters have been optimized for high precision and recall based on benchmarking to known ChIP-Seq peaks. Some minor bias from restriction cut site distribution and the position of cut sites relative to ChIP peaks is observed. For the most accurate analysis, we recommend a ChIP-seq dataset derived from the same cell material and antibody combination as the HiChIP data. For the complete list of internally- and customer-validated antibodies and lot numbers please visit our website at www.arimagenomics.com/validated_antibodies/. To submit your Arima-HiChIP validated antibody to Arima's community-backed database, please contact Technical Support.

2.8. Optimal read length, sequencing depth, replicate recommendations, and quality control

Arima-HiChIP libraries must be sequenced in paired-end mode, and are compatible with Illumina® sequencing machines (e.g. MiSeq®, NextSeq®, HiSeq®, NovaSeq™) and a variety of read lengths. Arima generally recommends 2x150bp read length on the HiSeq® or NovaSeq™ instruments to optimize for sequencing throughput and Arima-HiChIP data alignment quality, although shorter read lengths (e.g. 2x50bp, 2x100bp) and lower throughput instruments can certainly be used.

Arima provides detailed recommendations for estimating the optimal Arima-HiChIP sequencing depth to produce robust and reproducible chromatin loop discovery using the MAPS (Juric, 2019) data analysis pipeline. The optimal sequencing depth depends on the number of reads that can be used to identify chromatin loops and the desired resolution of the chromatin looping analysis. The default resolution for chromatin loop discovery using MAPS is 5kb. The reads that can be used to identify chromatin loops, called "long-range Fragments In Peaks" (or FRIPs), are the subset of reads that are long-range intra-chromosomal (i.e. "cis") interactions with at least one read-end mapping to a 5kb genomic bin containing a ChIP-seq peak, and with an

interaction distance ranging from 10kb to 2Mb. The number of 5kb genomic bins containing ChIP-seq peaks varies by cell type and protein factor, making it useful to define the *normalized* number of long-range FRIPs per 5kb bin containing a ChIP-seq peak, termed "long-range FRIPs per peak bin". A "high confidence" set of chromatin loops from two biological replicates (defined as >70% loops identified in one replicate that are also found in the other replicate), can be obtained if the minimum number of long-range FRIPs per peak bin reaches 380 per biological replicate. In the absence of replicate Arima-HiChIP data, or if one can only obtain 380 long-range FRIPs per peak bin by combining replicate Arima-HiChIP data, one may still expect to identify biologically meaningful loops although with a potentially higher "false positive" rate. The **Arima-HiChIP QC Worksheet** is specifically designed to integrate several quality metrics from shallow sequencing of Arima-HiChIP libraries and predict the required number of Raw PE Reads in order to obtain 380 long-range FRIPs per peak bin for each biological replicate. Please contact Technical Support for additional guidance.

Lastly, it is important to note that each Arima-HiChIP library should pass the experimental QC checkpoints and shallow sequencing QC analyses *prior* to deep sequencing. Please refer to the **Arima-HiChIP QC Worksheet** to compute and populate these QC values throughout the Arima-HiChIP experimental and bioinformatics workflows.

2.9. How to cite Arima-HiChIP in publications

When citing the Arima-HiChIP protocol or kit, we recommend: "HiChIP data was generated using the Arima-HiC$^+$ kit (P/N A101020), according to the manufacturer's protocols".

Crosslinking – Cell Culture

Input: Cells collected from cell culture
Output: Crosslinked cells

Before you begin: The Arima-HiChIP workflow for mammalian cell lines begins with the harvesting and crosslinking of 12-15 million mammalian cells per biological replicate. Each Arima-HiChIP 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 *Arima-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. Harvest cells from cell culture using standard protocols and pellet cells by centrifugation.
2. Resuspend in cell culture media, obtain a cell count by hemocytometer or automated cell counting methods.
3. Transfer 12-15 million cells to be crosslinked into a new 15mL conical tube, pellet cells by centrifugation and remove supernatant.
4. Resuspend cells in 5mL of RT **1X PBS**.

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.

5. Add 286µL of **37% formaldehyde**, to bring the final formaldehyde concentration to 2%.
6. Mix well by inverting 10 times and incubate at RT for 10 min.
7. Add 460µL of **Stop Solution 1,** mix well by inverting 10 times and incubate at RT for 5 min.
8. Pellet cells by centrifugation.
9. Discard supernatant.
10. Resuspend cells in 1mL **1X PBS**.
11. Aliquot cells into several new 1.5mL tubes, two with 0.5 million cells per aliquot, and one with the remaining 11-14 million cells. Mix sample by inversion between aliquots.
12. Pellet cells in all aliquots by centrifugation.
13. Discard supernatant, leaving only the crosslinked cell pellets and no residual liquid.
14. 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 – Cryopreserved Cells

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.

1. Fill a 15mL conical tube with 4mL of **1X PBS**.
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 PBS. *Do not* centrifuge the cells to try and remove the cryogenic freeze media. The following step also assumes the cells are preserved in 1mL of cryogenic freeze media, and transferring the cells into the PBS will bring the total volume to 5mL. If the cells are not frozen in 1mL of cryogenic freeze media, adjust the volume of PBS so that the total sample volume after Step 3 will be 5mL.

3. Gently transfer cells, including the cryogenic freeze media, into the conical tube containing 4mL of **1X PBS**, bringing the total volume to 5mL.
4. Add 286µL of methanol-stabilized **37% formaldehyde**, bringing the final formaldehyde concentration to 2%.
5. Mix well by inverting 10 times and incubate at RT for 10 min.
6. Add 460µL of **Stop Solution 1,** mix by inverting 10 times and incubate at RT for 5 min.
7. Place sample on ice and incubate for 15 min.
8. Pellet cells by centrifugation and discard supernatant.
9. Resuspend cells in 1mL **1X PBS**.
10. Aliquot cells into several new 1.5mL tubes, two with 0.5 million cells per aliquot, and one with the remaining 11-14 million cells. Mix sample by inversion between aliquots.
11. Pellet cells in all aliquots by centrifugation.
12. Discard supernatant, leaving only the crosslinked cell pellets and no residual liquid.
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

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.

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 to 4°C and cool 10ml of 1x PBS on ice per sample.
2. Embed a mortar, with a pestle inside of it, and a 50mL conical tube onto a cooler of dry ice and allow to cool. Cool a spatula by placing it in the 50mL conical tube for later use.
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.
4. Pour liquid nitrogen into the mortar until the entire pestle tip is submerged. Transfer 200-600mg frozen large animal tissue into mortar containing liquid nitrogen. Allow liquid nitrogen to evaporate just enough for the tissue to stay submerged.
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* 5 min per sample and some tissue types may take longer. The goal is to pulverize until the tissue resembles a fine powder without visible chunks.

Note: Using cold-resistant gloves is highly recommended to handle the mortar and pestle.

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.
7. Carefully pour pulverized tissue and remaining liquid nitrogen from the mortar into the 50mL conical tube. Ensure the tube does not overflow with liquid nitrogen.
8. Using the cooled spatula from Step 2, transfer any remaining pulverized large animal tissue from the mortar into the 50mL conical tube.
9. Submerge 50mL conical tube into dry ice to keep all the pulverized animal tissue frozen.

Note: Do NOT cap the 15mL conical tube until all the liquid nitrogen has completely evaporated.

10. Allow liquid nitrogen in 50mL 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.

11. Remove sample tube from dry ice.
12. Incubate sample tube for 10 min on ice.
13. Add 5mL of cold **1X PBS** and resuspend via pipetting with a 1ml pipette.
14. Pellet sample by centrifugation at 1,000 x G at 4°C for 5 min.
15. Discard supernatant.
16. Add 3mL of **TLB1** and resuspend via pipetting with a 1ml pipette.
17. Incubate sample tube for 20 min at 4°C, occasionally mix by inverting. Proceed to steps 18 and 19 below during the incubation.
18. Prepare a fresh 50mL conical with a 40µm cell strainer on top, per sample.
19. Rinse the 40µm cell strainer with 5-10ml DI water, discard flow through.
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.
21. Rinse cell strainer with 2mL 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.
22. Tap the cell strainer on the top of the 50ml 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.*
23. Pellet sample by centrifugation at 1,000 x G at 4°C for 5 min. Reduce the slowdown speed of the centrifuge so that the pellet will not be disturbed when the centrifuge stops.
24. Discard supernatant, making sure not to disturb the pellet.
25. Resuspend sample in 1mL of **TLB2**, using a 1ml pipette.
26. Overlay 3mL of **SS** 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.
27. Pellet sample by centrifugation at 2,500 x G at 4°C for 5 min. Reduce the slowdown speed of the centrifuge so that the sucrose gradient will not be disturbed when the centrifuge stops.
28. Discard supernatant.
29. Resuspend sample in 1 mL of cold **1X PBS**.
30. Pellet sample by centrifugation at 2,500 x G at 4°C for 5 min.
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.

32. Resuspend sample in 5 mL of room temperature **1X PBS**.
33. Add 286µL of **37% formaldehyde**, to bring the final formaldehyde concentration to 2%.
34. Mix well by inverting 10 times and incubate at RT for 10 min. Mix the sample every 2-3 mins with gentle inversion.
35. Add 460µL of **Stop Solution 1,** mix well by inverting 10 times and incubate at RT for 5 min. Mix the sample every 2-3 mins with gentle inversion.
36. Place sample on ice and incubate for 15 mins.
37. Pellet sample by centrifugation at 2,500 x G at 4°C for 5 min.
38. Discard supernatant.
39. Resuspend sample in 3mL cold **1X PBS**.
40. Pellet sample by centrifugation at 2,500 x G at 4°C for 5 min.
41. Discard supernatant.
42. Resuspend sample in 1mL cold **1X PBS**, and transfer to a 1.5 ml microfuge tube.
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*.
44. Pellet all aliquots by centrifugation at 2,500 x G at 4°C for 5 min. Discard supernatant leaving behind only the sample pellet and no residual liquid.
45. Snap freeze the aliquot cell pellets on dry ice for 5 mins or liquid nitrogen for 1 min, then store at -80°C for up to 1 year.

Estimating Input Amount – Mammalian Cell Culture and Cryopreserved Cells

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.

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.

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

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

3. Add 20µL of ● **Buffer E**, mix gently by pipetting, and incubate at 55°C for 30 min.
4. Incubate at 68°C for at least 3 hr. 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. Incubate at RT for 10 min.

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.

6. Add 225µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.

7. Place sample against magnet, and incubate until solution is clear.
8. Discard supernatant. While sample is still against magnet, add 500µL of 80% ethanol, and incubate at RT for 1 min.
9. Discard supernatant. While sample is still against magnet, add 500µL of 80% ethanol, and incubate at RT for 1 min.
10. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
11. Remove sample from magnet, resuspend beads thoroughly in 50µL of **Elution Buffer**, and incubate at RT for 5 min.
12. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
13. Quantify sample using Qubit®. Use 2µL of DNA for the Qubit® assay. The total DNA yield corresponds to the amount of DNA obtained from 0.5 million mammalian cells.
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 15µg of DNA per Arima-HiChIP reaction. If an average of 2µg 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 (15µg of DNA). Additionally, please note that the crosslinked cell pellet for one Arima-HiChIP reaction should occupy no more than 20µL of volume in the sample tube. If the crosslinked cell pellet comprises 15µg of DNA but occupies greater than 20µL of volume, aliquot the cells into multiple Arima-HiChIP reactions such that the sum of the DNA input from all reactions is 15µg and each cell pellet occupies no more than 20µL of volume, or contact Technical Support for additional guidance.

Estimating Input Amount – Large Animal Protocol

Input: Pulverized Crosslinked Tissue
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 – 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.

1. Thaw the two 10mg 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.

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

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

3. Add 20µL of ● **Buffer E**, mix gently by pipetting, and incubate at 55°C for 30 min.
4. Incubate at 68°C for at least 3 hr. 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. Incubate at RT for 10 min.

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.

6. Add 225µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.
7. Place sample against magnet, and incubate until solution is clear.

8. Discard supernatant. While sample is still against magnet, add 500µL of 80% ethanol, and incubate at RT for 1 min.
9. Discard supernatant. While sample is still against magnet, add 500µL of 80% ethanol, and incubate at RT for 1 min.
10. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
11. Remove sample from magnet, resuspend beads thoroughly in 50µL of **Elution Buffer**, and incubate at RT for 5 min.
12. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
13. Quantify sample using Qubit®. Use 2µL of DNA for the Qubit® assay. The total DNA yield corresponds to the amount of DNA obtained from 0.5 million mammalian cells.
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 15µg of DNA per Arima-HiChIP reaction. If an average of 2µg of DNA was obtained from*10mg of original input material* as calculated in Step 13 then the sample yields 200ng of DNA per mg of input tissue. This value is highly variable between different tissue types do to the density of cells in the tissue and the how difficult the tissue is to dissociate. To achieve an input of 15ug, for this example, 75 mg of the original tissue mass must be input into each reaction. If 200mg 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 20µL of volume in the sample tube. If the crosslinked cell pellet comprises 15µg of DNA but occupies greater than 20µL of volume, aliquot the cells into multiple Arima-HiChIP reactions such that the sum of the DNA input from all reactions is 15µg and each cell pellet occupies no more than 20µL of volume, or contact Technical Support for additional guidance.

Input: Crosslinked cells containing 15µg of DNA
Output: Immunoprecipitated proximally-ligated DNA fragments

Overview: Arima-HiChIP begins with generating biotin-labelled proximally-ligated chromatin using Arima-HiC chemistry (Section 3.1). The proximally-ligated chromatin is then sheared using Covaris® (Section 3.2.1) or Diagenode® Bioruptor® (Section 3.2.2) 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.

3.1. Arima-HiC

Before you begin: The pellet of previously crosslinked cells or nuclei for one Arima-HiChIP reaction should occupy no more than 20µL of volume and should be devoid of any residual liquid. If the cell pellet occupies greater than 20µL of volume, aliquot the cells such that the sum of the DNA input from all reactions is between 10-15µg and each cell pellet occupies no more than 20µL 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℃ and 37℃ for these incubations to avoid prolonged waiting periods before and between heated incubations*.

Note: Steps 4, 12, and 14 require addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix tables.

1. Aliquot cells for the Arima-HiChIP reactions by resuspending the crosslinked cell pellet from above in a total volume of 1ml of 1x PBS. Visually measure the volume of the cell pellet (50 – 100 ⌠l) 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⌠l of volume, resuspend it in 950⌠l of cold 1x PBS.
2. Aliquot the amount of cells required for 15µg based on the Determining Input Sections above by taking the proportion of the cell pellet that corresponds to the amount of cells determined to yield 15µg. For instance, if the cell pellet has 10 million cells and the determining input calculated that 4 million cells is needed for each HiChIP reaction then remove 400⌠l from the resuspended cell pellet and place in a new 1.7ml. 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 cold PBS, subtracting the volume of the cell pellet. Make sure the sample is homogenous then remove the fraction of the material recommended from the determining input step.
3. Pellet the cell aliquot and the remainder of the resuspended crosslinked cell pellet by centrifugation at 1,000 X G for 5 mins at 4°C.
4. Remove the supernatant from the cell pellets.
5. Re-freeze the crosslinked cell pellet on dry ice or liquid nitrogen, and store at -80°C.

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

2. Add 24µL of **Conditioning Solution**, mix gently by pipetting, and incubate at 62°C for 10 min. If using a thermal cycler, set the lid temperature to 85°C.

3. Add 20µL of ● Stop Solution 2, mix gently by pipetting, and incubate at 37°C for 15 min. If using a thermal cycler, set the lid temperature to 85°C.

4. Add 12µL of a master mix containing the following reagents:

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer A	7µL	7.7µL	x	2	=	15.4µL
● Enzyme A1	1µL	1.1µL	x	2	=	2.2µL
● Enzyme A2	4µL	4.4µL	x	2	=	8.8µL
Total	**12µL**					26.4µL

Note: If sonication is to be performed on the same day as HiC, please prepare R2 Buffer (Appendix B), during the 60 min incubation in the step below. Also prepare CS Buffer and MR1 Buffer (Appendix C) if shearing chromatin using a Covaris® instrument, or R1 Buffer (Appendix D) if shearing chromatin using a Diagenode® Bioruptor® Pico instrument. Preview sub-sections 3.2 and 3.3 below prior to execution.

5. Mix gently by pipetting, and incubate at 37°C for 60 min. If using a thermal cycler, set the lid temperature to 85°C.

6. Transfer sample to a clean 1.5mL microfuge tube.

7. Pellet sample by centrifugation at 10,000 x G at 4°C for 10 min.

8. Carefully discard supernatant, without disturbing the sample pellet.

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

10. Pellet sample by centrifugation at 10,000 x G at 4°C for 10 min.

11. Carefully discard supernatant, without disturbing the sample pellet.

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

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

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

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer B	12µL	13.2µL	x	2	=	26.4µL
● Enzyme B	4µL	4.4µL	x	2	=	8.8µL
Total	**16µL**					35.2µL

15. Mix gently by pipetting, and incubate at RT for 45 min.

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

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer C	70µL	77µL	x	2	=	154µL
● Enzyme C	12µL	13.2µL	x	2	=	26.4µL
Total	**82µL**					180.4µL

17. Mix gently by pipetting, and incubate at RT for 15 min.
18. 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 19.
    a. 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.
19. If, the user intends to optimize shearing settings for specific samples, follow the instructions in Appendix F before proceeding with step 20. 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 20.
20. Mix gently by inversion, and then immediately transfer 10µL 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.
21. If sample is in a PCR tube or PCR plate, transfer sample to a clean 1.5mL microfuge tube.
22. Pellet sample by centrifugation at 10,000 X G at 4°C for 10 min.
23. Discard supernatant. Once completed, store sample on ice and immediately proceed to the appropriate *Chromatin Shearing and Antibody Binding* sub-section depending on use of a Covaris® (Section 3.2.1) or Diagenode® Bioruptor® Pico (Section 3.2.2) instrument.

3.1.1. 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. 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.

1. Add 130µL of cold **CS Buffer** to sample pellet, and resuspend by gentle pipette mixing.
2. Transfer 130µL of sample to a Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube
3. Shear each sample using the Covaris instrument. Always store samples on ice except when the sample is being sheared.
    a. If shearing was optimized using the instructions in Appendix F, apply the optimized settings determined for each specific sample type.
    b. 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
	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

4. Transfer sheared sample to a 1.5mL 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.

5. Transfer 10µL 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.
6. Preclear the chromatin by adding 30µL of **Protein A Beads** to a new 1.5mL tube for each sample.
7. Place **Protein A Beads** against magnet, and incubate until solution is clear.
8. Discard supernatant.
9. Remove **Protein A Beads** from magnet, resuspend **Protein A Beads** in 880µL of cold **MR1 Buffer**, and mix gently by pipetting. Do not vortex.
10. Add the remaining 120µL of sheared sample from step 4, to bring the total volume to 1mL. Mix gently by pipetting. Do not vortex.
11. Incubate for ≥1 hr at 4°C on a nutator, rotator, orbital shaker or equivalent device.

    a. Sample should rotate at 4ºC while completing section **3.3** of the protocol. If completion of the aliquot DNA purification in section 3.3 takes less than 1 hour, ensure samples rotate for 1 hour to ensure sufficient pre-clearing.

3.1.2. 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 3 should be exactly as indicated.

1. Add 110µL of cold **R1 Buffer** to sample pellet, and resuspend by gentle pipette mixing.
2. Incubate at 4°C for 20 min.
3. Transfer exactly 110µL of sample to a Diagenode® 0.65mL Microtube.
4. Shear sample using the Diagenode® Bioruptor® Pico instrument
    a. 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. Note: Vortexing and quickly spinning the sample is *critical* for good shearing profiles and reproducibility between samples.
    b. 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.
5. Transfer sheared sample to a 1.5mL tube. Gently flick or spin the Diagenode® 0.65mL 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.

6. Transfer 10µL of sheared sample into a new tube labelled "Shearing QC". Store the Shearing QC sample at -20°C until use in the *Quality Control* section, and proceed to the next step with the remaining sample.
7. Preclear the chromatin by adding 30µL of **Protein A Beads** to a new 1.5mL tube for each sample.
8. Place **Protein A Beads** against magnet, and incubate until solution is clear.
9. Discard supernatant.
10. Remove **Protein A Beads** from magnet, resuspend **Protein A Beads** in 900µL of cold **R1 Buffer**, and mix gently by pipetting. Do not vortex.
11. Add to the remaining 100µL of sheared sample from step 5 above, to bring the total volume to 1mL. Mix gently by pipetting. Do not vortex.
12. Incubate for ≥1 hr at 4°C on a nutator, rotator, orbital shaker or equivalent device.

a. Sample should rotate at 4°C while completing the next section of the protocol. If completion of the aliquot DNA purification in section 3.3 takes less than 1 hour, ensure samples rotate for 1 hour to ensure sufficient pre-clearing.

3.2. 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 3.1.20 and 3.2.1.5). 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.

1. Thaw all Ligation QC and Shearing QC aliquots.
2. Add 90µL **Elution Buffer** to each Ligation and Shearing QC aliquot, to bring the total volume to 100µL**.**

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.

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

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer D	6µL	6.6µL	x	2	=	13.2µL
● Enzyme D	14.3µL	15.7µL	x	2	=	31.4µL
Total	**20.3µL**					44.6µL

4. Add 11.4µL 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°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.

5. Add 120µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.
6. Place sample against magnet, and incubate until solution is clear.
7. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
8. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
9. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
10. Remove sample from magnet, resuspend beads thoroughly in 30µL of **Elution Buffer**, and incubate at RT for 5 min.
11. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
12. Quantify sample using Qubit®. Use 2µL of DNA for the Qubit® assay.
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 -20°C until use in the *Chromatin Fragmentation QC* section below (Section 4.2).

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.

3.3. 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:

***Mass of antibody**= Shearing Yield x Antibody Ratio*

The antibody ratio is provided in the table below and the Shearing Yield was obtained in step 3.2.12 above.

1. Remove pre-clearing samples from 4°C (step 3.1.1.11 or 3.1.2.12), and place against magnet, and incubate until solution is clear.
2. Transfer the supernatant (precleared chromatin) to a new 1.5mL 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 1μL/1μg.

3. Use the worksheet in the **Arima-HiChIP QC Worksheet** on the *ChIP Efficiency* tab under "Shearing QC Samples"
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.
    a. 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 μg of shearing yield by the ratio listed in the table on the next page.

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

**This ratio is the μg of antibody needed for each μg of Shearing Yield*

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

6. Immediately after setting up the overnight incubation in the prior step, proceed to the Bead Blocking sub-section (Section 3.5) directly below.

3.4. Bead Blocking

Before you begin: This sub-section prepares the Protein A beads that will be used in the Chromatin Immunoprecipitation sub-section (Section 3.4) 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.

1. Add 30µL of **Protein A Beads** to a new 1.5mL tube for each sample.
2. Place **Protein A Beads** against magnet, and incubate until solution is clear.
3. Discard supernatant.
4. Remove **Protein A Beads** from magnet, resuspend **Protein A Beads** in 500µL **R2 Buffer**, and mix gently by pipetting. Do not vortex.
5. Incubate at 4°C overnight on a nutator, rotator, orbital shaker or equivalent device.

3.5. Chromatin Immunoprecipitation (ChIP)

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 2 hours. Alternatively, the thermomixer can be placed in a cold room or refrigerator.

Note: Step 15 requires addition of several reagents in the same step. These reagents should be combined into master mixes following the master mix tables.

1. Set a thermomixer to 4°C or place in a cold room or refrigerator and allow to cool for at least 2hr for the thermomixer to reach 4°C.
2. Remove blocked **Protein A Beads** from 4°C incubation, place against magnet, and incubate until solution is clear.
3. Discard supernatant.
4. Remove **Protein A Beads** from magnet, add 1mL **antibody-bound chromatin** to the blocked **Protein A Beads**, and mix by pipetting until homogeneous.

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 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.

5. Incubate at 4°C for 4hr on a nutator, rotator, orbital shaker or equivalent device.
6. Place sample against magnet, and incubate until solution is clear.
7. Discard supernatant. Resuspend sample in 1mL **R1 Buffer**, mix thoroughly by pipetting, and incubate on a thermomixer at 4°C for 3 min. with 1000 rpm shaking.
8. Repeat Steps 6-7 two times using **R1 Buffer,** for a total of 3 **R1 Buffer** washes.
9. Repeat Steps 6-7 two times using **R3 Buffer.**
10. Repeat Steps 6-7 one time using **LC Buffer**.
11. Repeat Steps 6-7 two times using **LTE Buffer**.
12. Place sample against magnet, and incubate until solution is clear.
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 15 for reverse crosslinking and subsequent DNA purification.

14. Resuspend sample in 174µL **Elution Buffer** and transfer the resuspended sample to a LoBind 1.5mL microfuge tube**.** The resuspended sample can also be transferred into a PCR tube or plate for the completion of Steps 15-16 below.

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

15. Add 35.5µL of a master mix containing the following reagents:

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

16. Add 20µL 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°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.

Note: DNA Purification Beads (e.g. AMPure® XP Beads) should be warmed to RT 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.

17. If sample is in a PCR tube or PCR plate, transfer sample into a LoBind 1.5mL microfuge tube.
18. Add 230µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.
19. Place sample against magnet, and incubate until solution is clear.
20. Discard supernatant. While sample is still against magnet, add 700µL of 80% ethanol, and incubate at RT for 1 min.
21. Discard supernatant. While sample is still against magnet, add 700µL of 80% ethanol, and incubate at RT for 1 min.
22. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
23. Remove sample from magnet, resuspend beads thoroughly in 50µL of **Elution Buffer**, and incubate at RT for 5 min.
24. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.

Note: The following step utilizes 2µL 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 0.05ng/µL, or 2.5ng of immunoprecipitated DNA. If the Qubit reading is below the limit of detection, there is less than 2.5ng of immunoprecipitated DNA but we still recommend proceeding to library preparation, assuming other QC metrics (see *Quality Control* section below) pass.

25. Quantify sample using Qubit®. Use 2µL of DNA for the Qubit® assay.
26. Record the sample concentration in the **Arima-HiChIP QC Worksheet** on the *ChIP Efficiency* tab under "ChIP QC Samples".
27. If >100ng of DNA is available, transfer 10ng of sheared sample into a new tube labelled "ChIP QC" and store at -20°C until later use in the *Quality Control* section.
28. Store remaining sample at -20°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

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**.

4.1. 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.

1. Thaw the Ligation QC and Shearing QC aliquots. Thaw the ChIP QC aliquot if it has already been collected.
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.

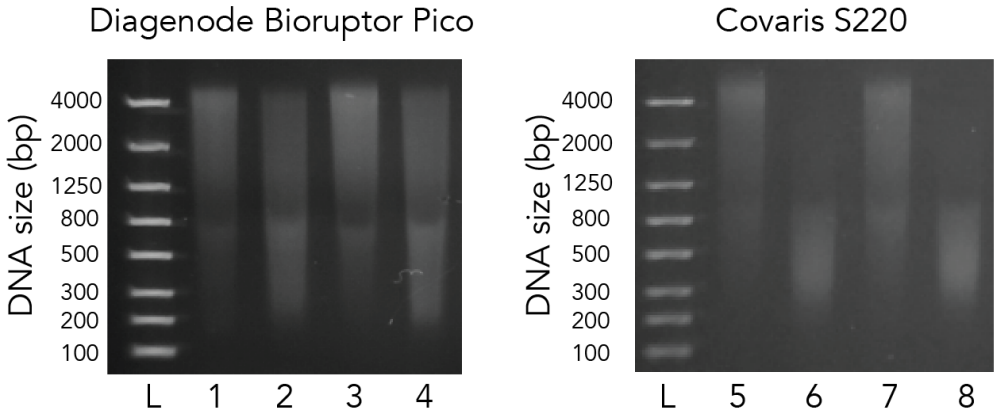


Fig.1 Expected DNA size distributions for proximally-ligated and sheared chromatin. Lonza 1.2% FlashGel electrophoresis analysis of DNA purified from proximally-ligated chromatin (Samples 1, 3, 5, and 7) and DNA purified after shearing using the Diagenode Bioruptor Pico instrument (Samples 2 and 4) and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

4.2. 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.

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 75ng of sample into a new tube labelled "Arima-QC1".
2. Add **Elution Buffer** to bring the volume to 50µL. The "Arima-QC1" sample should now contain 75ng of proximally-ligated DNA in 50µL of **Elution Buffer**.
3. Add 50µL of ● **QC Beads**, mix thoroughly by pipetting, and incubate at RT for 15 min.
4. Place sample against magnet, and incubate until solution is clear.
5. Discard supernatant, and remove sample from magnet.
6. Wash beads by resuspending in 200µL of **Wash Buffer**, and incubate at 55°C for 2 min.
7. Place sample against magnet, and incubate until solution is clear.
8. Discard supernatant, and remove sample from magnet.
9. Wash beads by resuspending in 200µL of **Wash Buffer**, and incubate at 55°C for 2 min.
10. Place sample against magnet, and incubate until solution is clear.
11. Discard supernatant, and remove sample from magnet.
12. Wash beads by resuspending in 100µL of **Elution Buffer**.
13. Place sample against magnet, and incubate until solution is clear.
14. Discard supernatant, and remove sample from magnet.
15. Resuspend beads in 7µL 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.

16. Quantify the total amount of *bead-bound DNA* using Qubit®. Use 2µL of thoroughly mixed bead-bound DNA for the Qubit® assay.
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.

Appendix A – Tissue Dissociation User-Supplied Buffers

Tissue Lysis Buffer 1 (TLB1), for 8 samples

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

* 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.

Tissue Lysis Buffer 2 (TLB2), for 8 samples

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**

* 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.

Sucrose Solution (SS), for 8 samples

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**

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

Appendix B – Day 1 Universal User-Supplied Buffers

R2 Buffer

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**

* 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.

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

Appendix C – Day 1 Covaris® User-Supplied Buffers

CS Buffer

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**

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

MR1 Buffer

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**

* 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

R1 Buffer

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**

* 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

R1 Buffer

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**

* 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.

R3 Buffer

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
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**

* 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.

LC Buffer

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**

* 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.

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*.

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**

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

Appendix F – Optional Shearing Titration

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.

1. Add 130µL of cold **CS Buffer** to sample pellet of the shearing optimization samples, and resuspend by gentle pipette mixing.
2. Transfer 130µL of sample to a Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube
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.

1 minute:

a. Begin by sonicating the shearing optimization sample using the shearing parameters provided below under "1 minute"

b. After completion of the 1 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "1 minute"

c. Add 10uL of CS buffer to the Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

3 minutes:

d. Sonicate the shearing optimization sample again using the shearing parameters provided below under "3 minutes"

e. After completion of the 3 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "3 minutes"

f. Add 10uL of CS buffer to the Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

5 minutes:

g. Sonicate the shearing optimization sample again using the shearing parameters provided below under "5 minutes"

h. After completion of the 5 minute sonication, transfer 10uL of the sheared sample to a new tube labeled "5 minutes"

i. Add 10uL of CS buffer to the Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

7 minutes:

j. Sonicate the shearing optimization sample again using the shearing parameters provided below under "7 minutes"

k. After completion of the 7 minute sonication, transfer 10uL of the sheared sample to a new tube labeled “7 minutes”

l. Add 10uL of CS buffer to the Covaris® microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm tube which contains the shearing optimization sample

9 minutes:

m. Sonicate the shearing optimization sample again using the shearing parameters provided below under “9 minutes”

n. After completion of the 9 minute sonication, transfer 10uL of the sheared sample to a new tube labeled “9 minutes”

i. Only 10uL is required for reverse crosslinking. The remaining 120uL can be discarded.

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

Setting	2% Crosslinked Cells				
	1	3	5	7	9
	Total Time (minutes)				
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.

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.

1. Thaw all shearing optimization aliquots.
2. Add 90µL **Elution Buffer** to each shearing optimization aliquot, to bring the total volume to 100µL**.**

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

3. Add 20.3µL of a master mix containing the following reagent:

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer D	6µL	6.6µL	x	5	=	33.0µL
● Enzyme D	14.3µL	15.7µL	x	5	=	78.5µL
Total	**20.3µL**					111.5µL

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

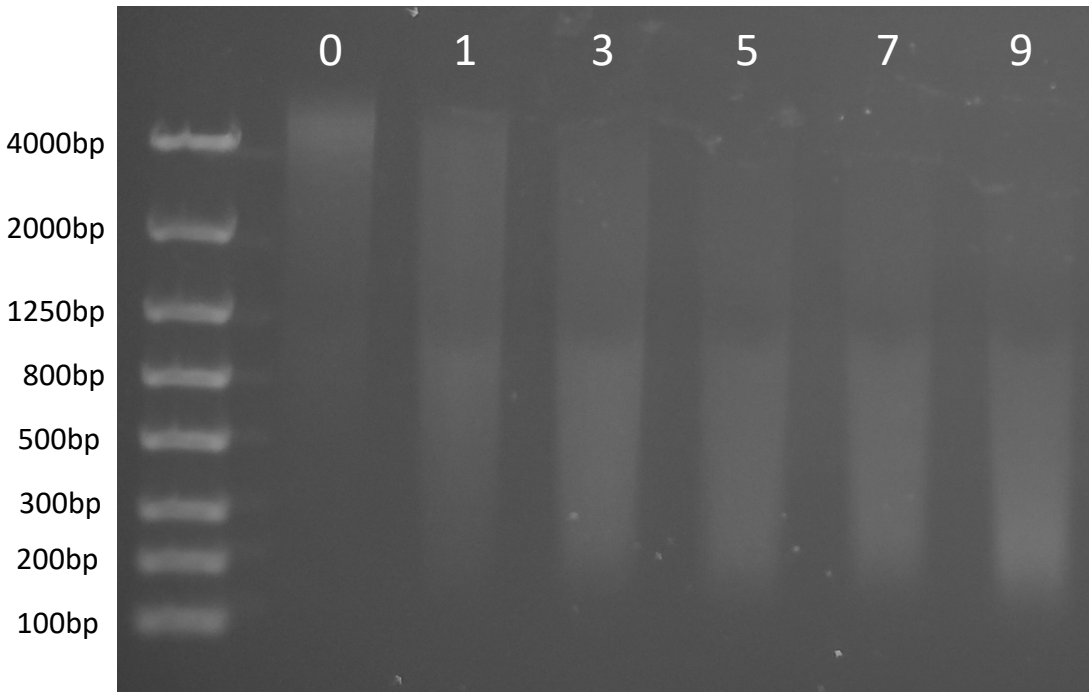
Temperature	Time
55°C	30 min.
68°C	90 min.*
25°C**	10 min.**

** 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.

5. Add 120µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.
6. Place sample against magnet, and incubate until solution is clear.
7. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
8. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
9. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
10. Remove sample from magnet, resuspend beads thoroughly in 30µL of **Elution Buffer**, and incubate at RT for 5 min.
11. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
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 FlashGelTM system are below.



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.

1. Add 110µL of cold **R1 Buffer** to sample pellet, and resuspend by gentle pipette mixing.
2. Incubate at 4°C for 20 min.
3. Transfer exactly 110µL of sample to a Diagenode® 0.65mL Microtube.

   10 Cycles:

4. Shear sample using the Diagenode® Bioruptor® Pico instrument using 30" ON / 30" OFF cycling conditions and 10 cycles total.
5. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "10 cycles"
6. Add 10uL of **R1** buffer to the Diagenode® 0.65mL Microtube which contains the shearing optimization sample
7. 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.

15 Cycles:

8. Shear sample using the Diagenode® Bioruptor® Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
9. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "15 cycles"
10. Add 10uL of **R1** buffer to the Diagenode® 0.65mL Microtube which contains the shearing optimization sample
11. 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.

20 Cycles:

12. Shear sample using the Diagenode® Bioruptor® Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
13. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "20 cycles"
14. Add 10uL of **R1** buffer to the Diagenode® 0.65mL Microtube which contains the shearing optimization sample
15. 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.

25 Cycles:

16. Shear sample using the Diagenode® Bioruptor® Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.
17. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled "25 cycles"
18. Add 10uL of **R1** buffer to the Diagenode® 0.65mL Microtube which contains the shearing optimization sample
19. 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.

30 Cycles:

20. Shear sample using the Diagenode® Bioruptor® Pico instrument using 30" ON / 30" OFF cycling conditions and 5 cycles total.

21. After completion of the 5 cycle sonication, transfer 10uL of the sheared sample to a new tube labeled “30 cycles”

    a. Only 10uL is required for reverse crosslinking. The remaining 120uL can be discarded.

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

1. Thaw all shearing optimization aliquots.
2. Add 90µL **Elution Buffer** to each shearing optimization aliquot, to bring the total volume to 100µL**.**

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

3. Add 20.3µL of a master mix containing the following reagent:

Reagent	Volume per reaction	10% extra		# reactions		Final
● Buffer D	6µL	6.6µL	x	5	=	33.0µL
● Enzyme D	14.3µL	15.7µL	x	5	=	78.5µL
Total	**20.3µL**					111.5µL

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

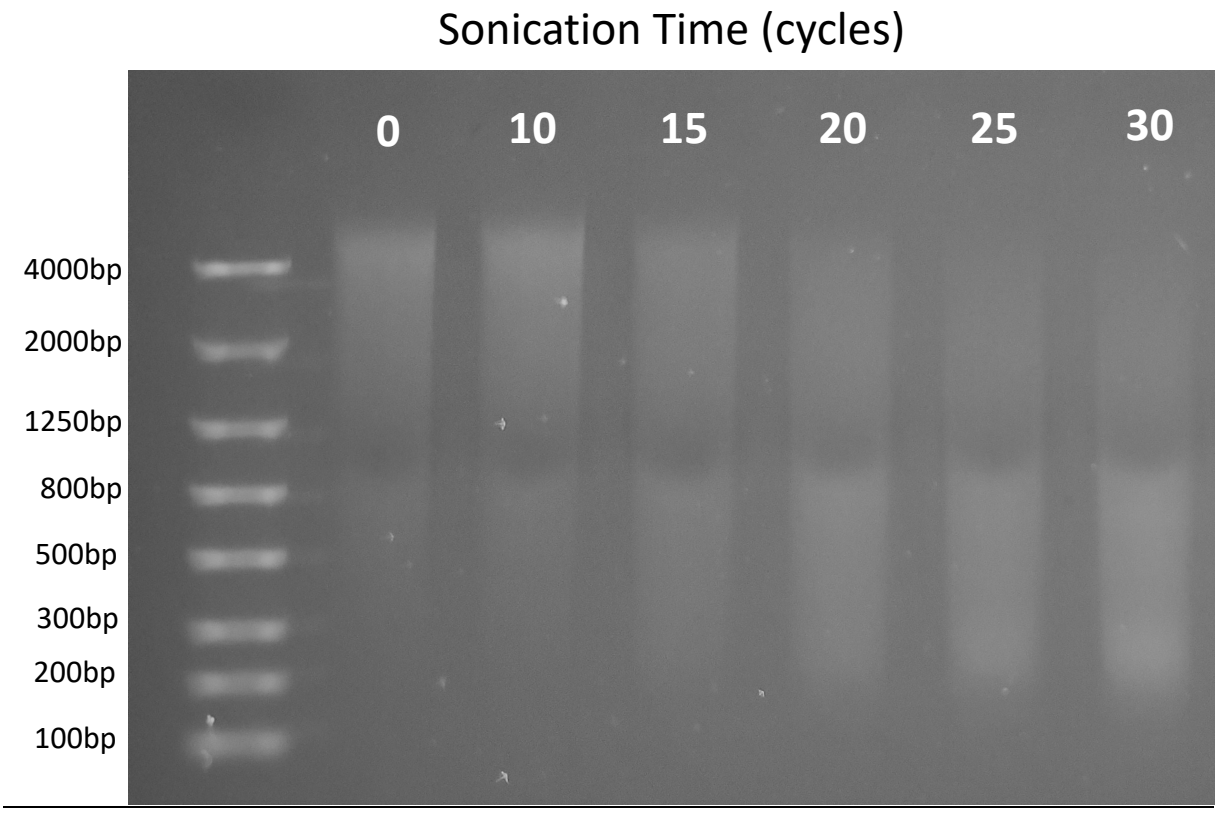
Temperature	Time
55°C	30 min.
68°C	90 min.*
25°C**	10 min.**

** 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.

5. Add 120µL of **DNA Purification Beads**, mix thoroughly, and incubate at RT for 5 min.
6. Place sample against magnet, and incubate until solution is clear.
7. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
8. Discard supernatant. While sample is still against magnet, add 200µL of 80% ethanol, and incubate at RT for 1 min.
9. Discard supernatant. While sample is still against magnet, incubate beads at RT for 3 – 5 min. to air-dry the beads.
10. Remove sample from magnet, resuspend beads thoroughly in 30µL of **Elution Buffer**, and incubate at RT for 5 min.
11. Place sample against magnet, incubate until solution is clear, and transfer supernatant to a new tube.
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 FlashGelTM. Exemplary results from the FlashGelTM system are below.



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CONTACT US

Technical Support: techsupport@arimagenomics.com
Order Support: ordersupport@arimagenomics.com