Video Article

# CARIP-Seq and ChIP-Seq: Methods to Identify Chromatin-Associated RNAs and Protein-DNA Interactions in Embryonic Stem Cells

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## Abstract

Embryonic stem (ES) cell self-renewal and differentiation is governed by extrinsic signals and intrinsic networks of transcription factors, epigenetic regulators, and post-translation modifications of histones that combinatorially influence the gene expression state of nearby genes. RNA has also been shown to interact with various proteins to regulate chromatin dynamics and gene expression. Chromatin-associated RNA immunoprecipitation (CARIP) followed by next-generation sequencing (CARIP-Seq) is a novel method to survey RNAs associated with chromatin proteins, while chromatin immunoprecipitation followed by next-generation sequencing (ChIP-Seq) is a powerful genomics technique to map the location of post-translational modification of histones, transcription factors, and epigenetic modifiers on a global-scale in ES cells. Here, we describe methods to perform CARIP-Seq and ChIP-Seq, including library construction for next-generation sequencing, to generate global chromatin-associated RNA and epigenomic maps in ES cells.

## Video Link

The video component of this article can be found at https://www.jove.com/video/57481/

## Introduction

Embryonic stem (ES) cell fate decisions are regulated by communication between extracellular signals and a host of transcriptional-regulators, including histone modifiers, and post-translation modification of histone tails. These interactions facilitate chromatin accessibility and packaging of chromatin into one of two states: euchromatin, which is open and transcriptionally active, and heterochromatin, which is compact and generally transcriptionally inactive. Transcription factors with DNA sequence-specific binding affinities and epigenetic modifiers associate with euchromatic regions to participate in controlling gene expression. Next-generation sequencing methods, including ChIP-Seq[1], have been instrumental in mapping genome-wide transcriptional networks that are fundamental for ES cell self-renewal and pluripotency[2,3,4,5,6]. Moreover, while RNA immunopreciation followed by next-generation sequencing (RIP-Seq)[7] evaluations of RNA-protein interactions suggest that DNA binding proteins interact with RNAs to regulate transcriptional events[7,8,9,10,11,12], few studies have investigated the genome-wide localization of RNAs associated with chromatin[12], or global interactions between RNA and histone modifications. Long non-coding RNAs (lncRNAs) are one class of RNAs which have been found to regulate the activity of chromatin-associated proteins[13,14,15]. For example, Xist is a lncRNA that regulates, in female mammalian cells, inactivation of one X chromosome, through the recruitment of epigenetic repressors[16,17]. However, the full spectrum of RNAs associated with chromatin is largely unknown. Here, we describe a novel protocol, chromatin-associated RNA immunoprecipitation (CARIP) followed by next-generation sequencing (CARIP-Seq), to identify chromatin-associated RNAs on a genome-wide basis in ES cells, including library preparation for next-generation sequencing, and ChIP-Seq to map global occupancy of histone modifications, transcription factors, and epigenetic modifiers. Unlike other RIP-Seq methods[7], CARIP-Seq includes crosslinking and sonication steps, which allow for the direct identification of RNAs associated with chromatin. Together, ChIP-Seq is a powerful tool to identify genome-wide protein-DNA interactions, while CARIP-Seq is a powerful method to survey RNAs associated with chromatin components.

## Protocol

### 1. Culture of Mouse ES Cells in Feeder-Free Conditions.

NOTE: Mouse ES cells are conventionally cultured in media on a cell culture dish coated with gelatin and a mono-layer of mouse embryonic fibroblasts (MEF), which have been mitotically inactivated (iMEFs). However, MEFs should be removed prior to downstream epigenetic or expression analyses to prevent the contamination of MEF-associated chromatin and RNA.

1. Preparation of a feeder layer: Gelatin coat a 6-well cell culture plate by adding 2 mL of 0.1% gelatin in water, and incubate the plate at 37 °C for 20-30 min.
2. Prepare 10% DMEM high-glucose media containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), and 1x penicillin/streptomycin.
3. Isolate MEFs from E13.5 to E14.5 mice[18,19] or acquire from a commercial vendor. Mitotically inactivate MEFs by treating with mitomycin C or gamma irradiation using standard protocols[19]. Alternatively, mitotically inactivated MEFs (iMEFs) can be acquired from a commercial vendor.
4. Culturing MEFs. Pre-warm MEF media at 37 °C, thaw frozen vial of iMEFs and culture in 10% FBS MEF media at 37 °C with 5% $CO_2$. Plate MEFs at ~200,000 cells per well of a 6-well culture dish.
5. Culturing ES cells on a layer of feeder cells (iMEFs).
    1. 12-24 h after plating iMEFs, pre-warm a 50 mL tube of ES cell media (DMEM high-glucose, LIF (10 ng/mL), 15% ES cell-qualified FBS, 1× cell-culture qualified 2-mercaptoethanol, 1× glutamine, nonessential amino acids (NEAA), 1× penicillin/streptomycin), at 37 °C with 5 % $CO_2$.
    2. Aspirate the media from the cell culture plates containing iMEFs, re-suspend the ES cells in 2 mL of media (after thawing at 37 °C), and plate the cells on the layer of iMEFs. When placing the culture dish in the incubator, it is important to move the dish in a "t" shape to evenly distribute the cells and to prevent aggregation towards the center of the dish.
6. Passage the ES cells onto feeder-free gelatin-coated dishes. Passage the ES cells before they become confluent. ES cell colonies maintain self-renewal best if the colonies are evenly spaced and not confluent. The presence of many ES cell colonies that are touching indicates that the cell density is too high.
    1. Coat a 6-well culture dish with gelatin as described above. Incubate 0.25% trypsin-EDTA at 37 °C for ~10-15 min to pre-warm trypsin.
    2. Next, passage the ES cells by first washing with 1x phosphate buffered saline (PBS) and adding 1 mL of 0.25% trypsin-EDTA solution. Wait 1-2 min for the colonies to detach. Dissociate the colonies to a single-cell suspension by pipetting with a 1 mL tip for ~5 times.
    3. Use a microscope to confirm single cell dissociation of the ES cells. To quench the reaction, or to "neutralize" the trypsin, add 10% FBS MEF media. Centrifuge the cells at 300 x g at room temperature for 3-5 min, and aspirate the media.
    4. Re-suspend the cellular pellet in 2 mL of media (ES cell) supplemented with glycogen synthase kinase-3 (GSK3) inhibitor (CHIR99021; GSK3i), or 2i (MEKi/GSK3i) conditions (2-3 µM GSK3i: CHIR99021, 1 µM MEKi: PD0325901).
    5. Aspirate the gelatin from the cell culture dish, and add 2 mL of media containing ESCs to the dish, and place the dish in the 37 °C incubator (**Figure 1A-D**). Dual inhibition of GSK3i and MEKi promotes ground state naïve pluripotency of ES cells in the absence of feeder cells (iMEFs) and without serum[20]. ES cells should be passaged two to three times to remove MEFs before proceeding to crosslinking.

## 2. Crosslinking of ES Cells for ChIP and CARIP

1. Harvest the ES cells by washing them with PBS, then add 0.25% trypsin pre-warmed at 37 °C. Incubate the cells for several minutes at room temperature. Avoid over trypsinization of ES cells, which will lead to cell death. Use a microscope to evaluate single cell dissociation.
2. Quench trypsin digestion as described above for passaging ES cells, and centrifuge the cells at 300 x g at room temperature for 3-5 min.
3. Re-suspend the cells in pre-warmed (37 °C) MEF media (10% FBS/DMEM) at 2 × $10^6$ cells/mL.
4. To crosslink the cells, add 37% formaldehyde solution to a 1% final concentration, and incubate the cells in a conical tube (15 mL or 50 mL) at 37 °C for 8 min. Invert the tube several times during the 8 min period to achieve homogenous fixation.
   NOTE: The fixation time can be empirically optimized for CARIP. If the fixation time is altered, the number of sonication cycles should also be adjusted. Also, the 37 °C temperature increases crosslinking efficiency compared to crosslinking at room temperature. The temperature of fixation can be empirically optimized.
5. To stop the fixation reaction, add 1.25 M glycine (pre-chilled at 4 °C) to a concentration of 0.125 M. Invert the tube at 8 rpm using a rotator for 5 min at room temperature. Centrifuge the sample at 300 x g for 5 min at room temperature and aspirate the media.
6. Wash the cell pellet with pre-chilled PBS (4 °C), centrifuge the sample at 300 x g for 5 min at room temperature, and aspirate PBS. Wash the cells with PBS again. Next, aliquot 15 × $10^6$ cells per 15 mL tube.
7. Freeze the cell pellet by placing it in liquid nitrogen for 1 min, on dry ice for 5 min, or place immediately at -80 °C.
   NOTE: Fixed ES cell pellets can be stored for up to 6 months without decreased quality in downstream experiments.

## 3. Chromatin sonication for ChIP and CARIP

1. Thaw fixed ES cell pellet on ice. Re-suspend the cell pellet in sonication buffer (2.5 mL). For transcription factors or epigenetic regulators (protein factors), re-suspend the pellet in 1x TE, 1mM PMSF, 1x proteinase inhibitor cocktail. For histone modifications, re-suspend the pellet in 1x TE, 0.1% SDS, 1mM PMSF, 1x proteinase inhibitor.
   NOTE: While the addition of SDS enhances sonication efficiency, it may not be beneficial for evaluating certain chromatin constituents or transcription factors.
2. For chromatin immunoprecipitation (ChIP), sonicate chromatin with 14-18 cycles (30 s on, 30 s rest) at 40% amplitude. For chromatin-associated RNA immunoprecipitation (CARIP), reduce the number of sonication cycles (8-10 cycles). Due to cellular, fixation, and sonifier variability, conditions for sonication should be empirically determined.
3. Supplement the sonication buffer with 28 µL of 10% SDS for protein-factors, 28 µL of sodium-deoxycholate, and 0.28 mL of 10% Triton-X100, and mix well.
   NOTE: Addition of these components to the sonication buffer generates RIPA buffer.
4. Centrifuge the sample at 10,000 x g for 10 min at 4 °C to pre-clear the chromatin. Move the supernatant to a fresh tube and proceed with chromatin immunoprecipitation.
5. Alternatively, sonicated chromatin can be stored at -80 °C until use. However, the storage of sonicated chromatin at -80 °C may lead to diminished quality for protein-factor ChIP.

## 4. ChIP and CARIP Process

1. Add 40 µL of protein A or G magnetic beads to a 1.5 mL, low binding tube. Add 600 µL of PBS to wash the beads. Place the tube onto a rack containing a magnet, incubate the tube for 1 min at room temperature , remove PBS, and re-suspend the magnetic beads in 100 µL of PBS.
2. Add 2-4 µg of antibody to the tube with magnetic beads. Incubate and rotate the tube at room temperature for 40 min at 8 rpm using a rotator to facilitate the antibody binding to the magnetic beads. Insert the tube onto the magnetic rack, incubate it for 1 min at room temperature, then remove PBS.
3. Add 200 µL of PBS to wash the magnetic beads. Washing with PBS is performed to remove free IgGs. Incubate and rotate the tube at room temperature for 5 min between wash steps. Attach the tube to the magnet and remove the PBS.
4. Immunoprecipitate chromatin-bound proteins by incubating the sonicated chromatin extract ($4 \times 10^6$ cells per ChIP), with the magnetic beads and rotate the sample at 4 °C (overnight).
5. Wash the magnetic beads. Incubate the sample at room temperature with the following buffers and rotate the sample for 10 min with each buffer: Twice with 1 mL of RIPA buffer (TE (10 mM Tric-HCl, 1 mM EDTA) pH 8.0, 1% Triton X-100, 0.1% sodium deocycholate, 0.1% SDS), twice with 1 mL of RIPA buffer containing 0.3 M NaCl, twice with 1 mL of LiCl buffer (0.5% sodium deocycholate, 0.5% NP40, 0.25 M LiCl), once with 1 mL of TE buffer with 0.2% Triton X-100, and once with 1 mL of TE.
6. De-crosslinking for ChIP. Re-suspend the magnetic beads in 100 µL of TE. Add 3 µL of 10% SDS and 5 µL of proteinase K (20 mg/mL). Incubate the sample at 65 °C (overnight).
   NOTE: It is important to cover the tubes during this step with parafilm or plastic wrap to prevent evaporation.
   1. The following day, invert the tube several times to mix. Next, use the magnet rack to transfer the supernatant to a fresh tube. To wash the magnetic beads, add 100 µL of TE containing 0.5 M NaCl, and subsequently combine the two supernatants.

7. De-crosslinking for CARIP. Re-suspend the magnetic beads in 100 µL of TE. Add 3 µL of 10% SDS and 5 µL of proteinase K (20 mg/mL). Incubate the sample at 65 °C for 4 to 12 h. Incubation time at 65 °C needs to be empirically determined due to variability between cell types, sonication and fixation conditions, etc.
   1. Invert the tube 3-5 times to mix, apply the tube to the magnetic rack, and subsequently move the supernatant to a fresh tube.
   2. To wash the magnetic beads, add 100 µL of TE containing 0.5 M NaCl, and subsequently combine the two supernatants.
      NOTE: It is important to use nuclease-free reagents (*e.g.*, TE buffer, SDS, *etc.*) to prevent the loss of DNA or RNA during the de-crosslinking step.

8. Extract DNA/RNA using 200 µL of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v).
   NOTE: RNA can also be extracted using commercial kits using the manufacturer's instructions. Shake for 30 s, spin at 10,000 x g at room temperature for 10 min, and move supernatant to a fresh tube.
   1. To precipitate DNA/RNA, add 2 µL of GlycoBlue, 20 µL of 3 M sodium acetate, and 600 µL of ethanol. Mix by inverting ~5-8 times, and place the sample on dry ice or at -80 °C for at least 1-2 h.
   2. Centrifuge the sample at 10,000 x g for 30 min using a refrigerated (4 °C) table-top centrifuge. Air dry the pellet for <1 min and re-suspend it in 40 µL of EB buffer (10 mM Tris-HCl). For ChIP-Seq library preparation, continue to step 6.

9. Remove DNA from chromatin-associated RNA IP samples using DNase. Add 10x DNase buffer to 1x concentration in the CARIP sample. Add 1 µL of DNase for up to 10 µg of RNA (50 µL reaction). Incubate the sample at 37 °C for 30 min. Extract the RNA sample with PCI. Resuspend in nuclease-free $H_2O$.

## 5. Double-stranded cDNA Synthesis for CARIP-Seq

1. Synthesis of first-strand cDNA. For the reverse-transcription reaction, set up the following reaction in a tube designed for a PCR machine: 10.5 µL of RNA and 1 µL random of hexamer primers (3 µg/µL).
2. Incubate the tube at 65 °C for 5 min to denature the secondary structure, and store it on ice for 2 min. Mix the following components: 4 µL of first-strand buffer, 2 µL of 0.1 M DTT, 1 µL of dNTP, and 0.5 RNase OUT. Next, to the PCR tube, add 7.5 µL of the master mix. Incubate the tube for 2 min at 25 °C. Add 1 µL of Superscript II (200 U/µL) and incubate as follow: 25 °C for 10 min, 42 °C for 50 min, 70 °C for 15 min, and hold at 4 °C.
3. Second-strand cDNA synthesis. Place the tube on ice. Next, add the following components in order: 91 µL of diethyl pyrocarbonate (DEPC) treated water, 30 µL of 5x second-strand reaction buffer, 3 µL of dNTP mix (10 mM), 1 µL of *E. Coli* DNA ligase (10 U/µL), 4 µL of *E. Coli* DNA Polymerase (10U/µL), and 1 µL of *E. Coli* RNase H (2U/µL). Mix by gently vortexing and incubate at 16 °C for 2 h.
   NOTE: Mixing by flicking or inverting the tube may not be sufficient to thoroughly mix the reagents.
4. Add 2 µL of T4 DNA Polymerase and incubate at 16 °C for 5 min. Purify cDNA (double-stranded, dscDNA) using PCR purification kit. Elute the dscDNA in 40 µL of EB buffer.
5. Fragment double-stranded cDNA. Shear cDNA (dscDNA) using a water bath sonifier and a 1.5 mL tube to a size of 250-500 bp. Sonication of dscDNA is critical to reduce fragment sizes to facilitate sequencing of the entire RNA transcript.
6. When using a standard model of a water-bath sonifier, sonicate the sample for 3× 10 min, or 4× 10 min.
   NOTE: Three cycles should be used for up to 1 µg of total RNA, while four cycles are recommended for 1-5 µg of total RNA. Centrifuge the tube briefly after each sonication cycle, and maintain a cool water bath by adding fresh ice.
7. When using a Pico sonifier, it is recommended to use six cycles for 1-5 µg of total RNA and the following cycling conditions (30 s on, 90 s off). Centrifuge the tube briefly after 2-3 sonication cycles. The efficiency of sonication can be determined by loading a fraction of the sheared dscDNA (3-4 µL) on an agarose gel (2%). It is important to empirically test sonication conditions due to variability between sonifiers.
8. Perform library preparation as described in step 6 for next-generation sequencing.

## 6. ChIP-Seq and CARIP-Seq Library Construction

1. Repair ends of DNA using a DNA end-repair kit. This kit is used to generate blunt-ended DNA. For this reaction, set up the following in a 1.5 mL, low binding tube: 34 µL of DNA, 5 µL of 2.5 mM dNPTs, 5 µL of 10 mM ATP, 5 µL of 10x end-repair buffer (5 mM DTT, 100 mM magnesium acetate, 660 mM potassium acetate, 300 mM Tris-acetate, pH 7.8), and 1 µL of end-Repair Enzyme mix (T4 polynucleotide kinase, T4 DNA polymerase).
2. Incubate the sample for 45 min at room temperature.
3. Purify DNA using a reaction cleanup kit. Elute end-repaired DNA in 32 µL of pre-warmed (65 °C) EB buffer. While we do not usually quantitate the concentration of DNA following ChIP, or RNA following CARIP, prior to library preparation, it is recommended to start with at least 5-10 ng of ChIP DNA or CARIP RNA.
4. Addition of a 3' "A" overhang to end-repaired DNA. Add the components to a 1.5 mL, low binding tube: 30 µL of DNA from above, 5 µL of 10x NEB buffer 2, 1 µL of dATP from 10 mM stock, 11 µL of $H_2O$, and 3 µL of 5 U/ µL Klenow fragment (3'-5' Exo-), and mix gently by pipetting.
5. Incubate the sample for 30 min at 37 °C.
6. Use a reaction cleanup kit to purify DNA. Elute DNA in 25 µL of pre-warmed (65 °C) EB buffer.
7. Ligate adapter. Add the following components on ice: 23 µL of DNA from above, 3 µL of 10x T4 DNA ligase buffer, 1 µL of annealed PE or multiplexing adapter (4 µM), and 3 µL of T4 DNA ligase (400 U/µL), and mix gently by pipetting.
   NOTE: Adapter sequences may also be obtained from a commercial source.
   **Oligonucleotide sequences:**
   **PE Adapters**
   5' P-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
   5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
   **Multiplexing Adapters**
   5' P-GATCGGAAGAGCACACGTCT
   5' ACACTCTTTCCCTACACGACGCTCTTCCGATCT
8. Incubate the sample for 30 min at room temperature.
9. Perform size selection of DNA using a pre-cast (2%) agarose gel. Run the gel for 10 min.
10. Excise the region corresponding to 250-450 bp.
    NOTE: By cutting the gel above above 200 bp, there is a decreased possibility of contamination from unligated adapter dimers. It is important to remove any unligated adapters prior to the PCR step.
11. Use a gel extraction kit to purify DNA. Elute in 20 µL of EB buffer.
12. PCR and library purification for ligated DNA samples containing paired-end (PE) adapters. Add the components in a PCR tube: 12 µL of 50% of DNA from above, 12 µL of water, 25 µL of master mix (Phusion HF), 1 µL of PE PCR primer 1.0, and 1 µL of PE PCR primer 2.0.
    NOTE: Oligonucleotide sequences may also be obtained from a commercial source.
    **Oligonucleotide sequences:**
    **PE PCR Primer 1.0**
    5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
    **PE PCR Primer 2.0**
    5' CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATCT
13. PCR and library purification for ligated samples containing Index PE adapters. Add the components: 12 µL of 50% of DNA from above, 12 µL of water, 1 µL of PCR primer InPE 1.0 (diluted 1:2), 1 µL of PCR primer InPE 2.0 (diluted 1:2), and 1 µL of PCR primer Index (diluted 1:2).
    NOTE: oligonucleotide sequences may also be obtained from a commercial source.
    **Oligonucleotide sequences:**
    **Multiplexing PCR Primer 1.0**
    5' AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
    **Multiplexing PCR Primer 2.0**
    5' GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
    **PCR Primer Index Sequences 1 - 12**
    **PCR Primer, Index 1**
    5' CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTC
    **PCR Primer, Index 2**
    5' CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTC
    **PCR Primer, Index 3**
    5' CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTC
    **PCR Primer, Index 4**
    5' CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTC
    **PCR Primer, Index 5**
    5' CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTC
    **PCR Primer, Index 6**
    5' CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTC
    **PCR Primer, Index 7**
    5' CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTC
    **PCR Primer, Index 8**
    5' CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTC
    **PCR Primer, Index 9**
    5' CAAGCAGAAGACGGCATACGAGATCTGATCGTGACTGGAGTTC
    **PCR Primer, Index 10**
    5' CAAGCAGAAGACGGCATACGAGATAAGCTAGTGACTGGAGTTC
    **PCR Primer, Index 11**
    5' CAAGCAGAAGACGGCATACGAGATGTAGCCGTGACTGGAGTTC

**PCR Primer, Index 12**
5' CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTC

14. Perform PCR cycling using the following conditions (denature for 30 s at 98 °C, 18 cycles of 10 s at 98 °C, 30 s at 65°C, 30 s at 72 °C, 5 min at 72 °C, hold at 4 °C).
    NOTE: The number of PCR cycles should be determined empirically.
15. Size selection of PCR products. Run DNA on a pre-cast E-Gel EX (2%) or homemade 2% gel (agarose) and excise the 300 - 500 bp region.
16. Use a gel extraction kit to purify the DNA from the gel. Elute final PCR product in 20 µL of EB buffer.
17. Use a fluorometer to measure the concentration of the library. Perform next-generation sequencing.

## Representative Results

We successfully interrogated the genome-wide binding of H3K4me3, H3K4me2, and KDM5B in ES cells using this ChIP-Seq protocol[6]. ES cells were cultured in feeder-free conditions (**Figure 1**), and cross-linked as described above. Sonication was subsequently performed as described in step 3.2 of the ChIP-Seq protocol, and evaluated by running DNA on a 2% agarose gel (**Figure 2**). Next, ChIP was performed as described in step 4 and library preparation was performed as described in step 6. ChIP-Seq libraries were sequenced on a next-generation sequencing platform. Representative custom UCSC browser views reveal enrichment of H3K4me3, H3K4me2, and KDM5B at the core pluripotency gene *POU5F1* (**Figure 3A**), and at the HOXC cluster (**Figure 3B**). CARIP-Seq was also performed by following the protocol as described in steps 4-6. A representative UCSC browser view reveals enrichment of H4K20me3-associated RNA (CARIP-Seq) and H4K20me3 occupancy (ChIP-Seq) at a loci in ES cells (**Figure 4**). The CARIP-Seq signal demonstrates that while the RNA is associated with H4K20me3, it does not necessarily indicate that it interacts with the locus shown. CARIP-Seq datasets can be analyzed in a similar way as ChIP-Seq datasets. Data generated from mouse ES cell CARIP-Seq and ChIP-Seq experiments can both be aligned to a reference genome (*e.g.*, mm9, mm10) using bowtie2[21]. ChIP-Seq and CARIP-Seq enriched regions can both be identified using peak calling programs such as "Spatial Clustering for Identification of ChIP-Enriched Regions" (SICER)[22] or MACS[23,24]. Because CARIP-Seq datasets likely include broad peaks, it is important to use an algorithm that can identify broad and sharp peaks. While the protocols described in this manuscript have been optimized for performing ChIP-Seq and CARIP-Seq using ES cells, these protocols should also be suitable for evaluating protein-DNA and chromatin-associated RNA interactions in other cell types.

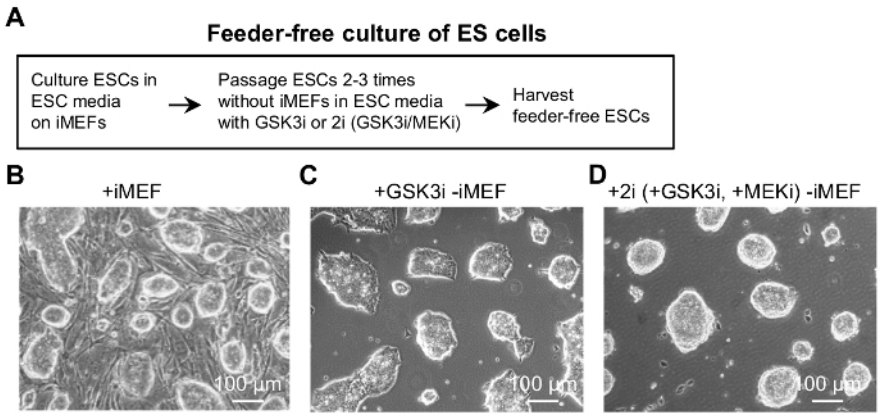


**Figure 1: Feeder-free culture of ES cells.** (A) Experimental design. (B) ES cells cultured on iMEFs in ES cell media containing LIF, or in feeder-free conditions with ES cell media containing LIF and (C) GSK3i or (D) GSK3i and MEKi (2i). Please click here to view a larger version of this figure.

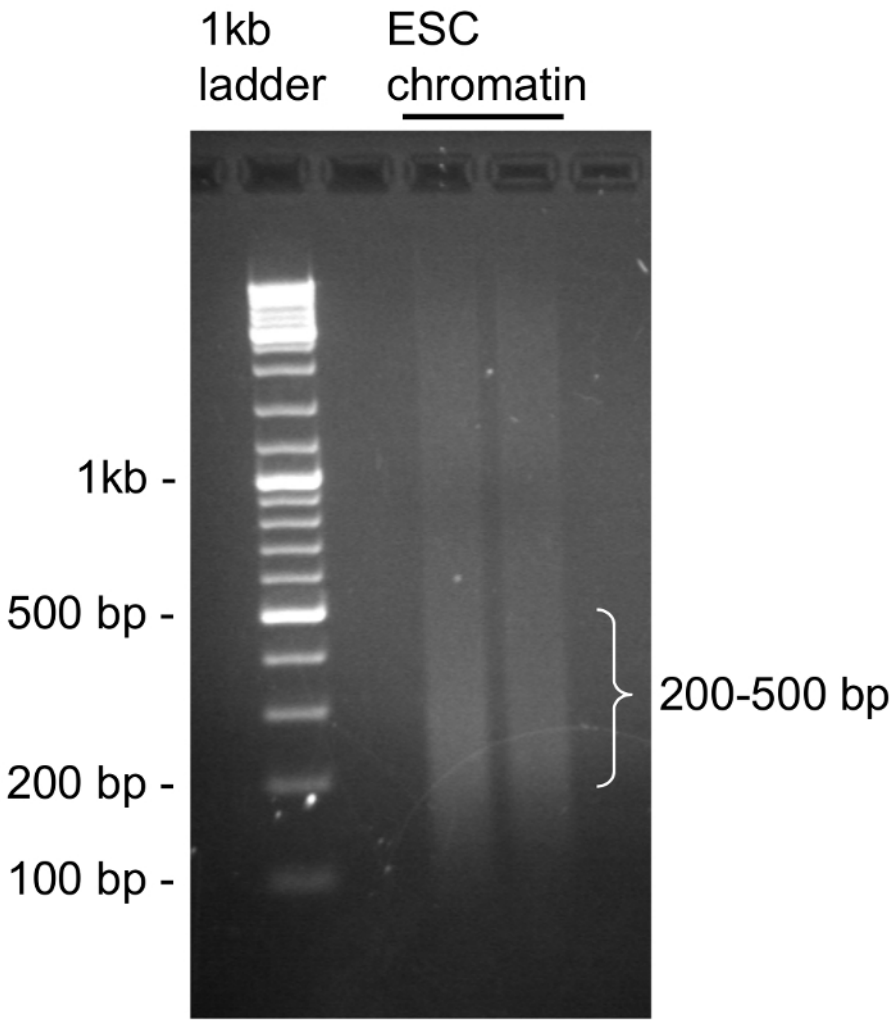


**Figure 2: Sonication of crosslinked ES cell chromatin.** Crosslinked ES cells were sonicated using a sonifier with a microtip for 18 cycles (40% amplitude, 30 s on, and 30 s rest). Crosslinking was reversed, and RNase digestion was subsequently performed to remove RNA. DNA was purified using PCI and run on an agarose gel (2%). Please click here to view a larger version of this figure.

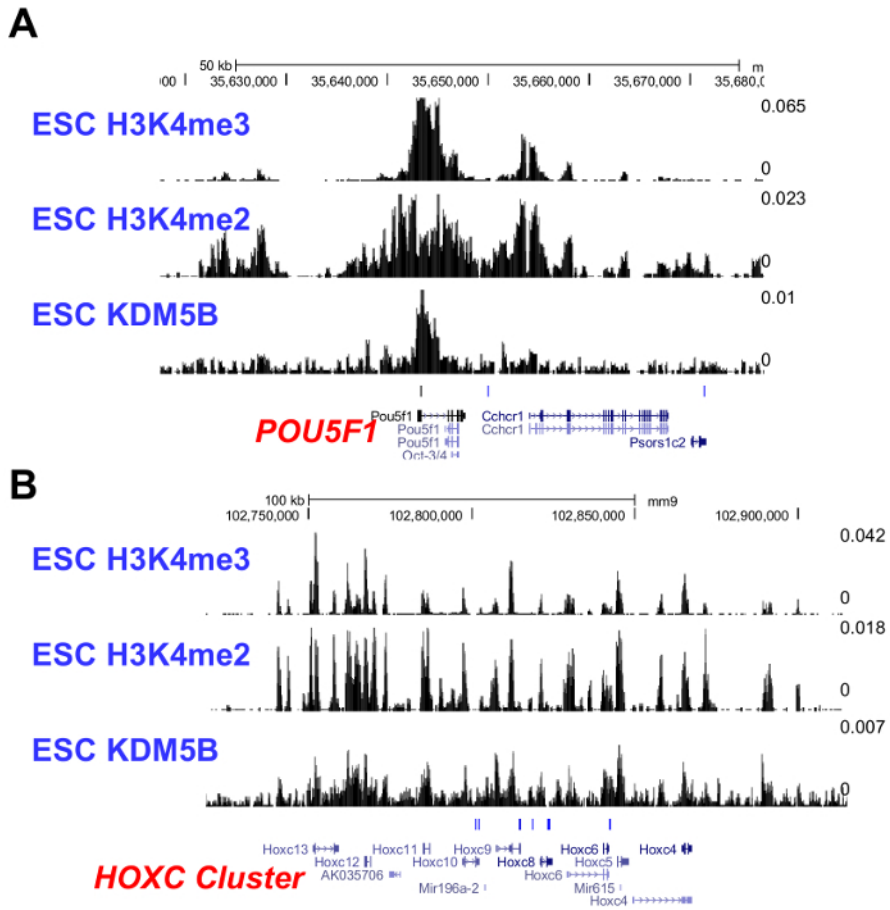


**Figure 3: Representative ChIP-Seq analysis of a histone modifying enzyme and histone modifications in ES cells.** Custom UCSC browser views of H3K4me3, H3K4me2, and KDM5B at the pluripotency-regulator (A) POU5F1 and the (B) HOXC cluster in ES cells (normalized tag density (RPBM), read per base per million reads). Note the overlap between KDM5B binding and H3K4me3/2 occupancy. Please click here to view a larger version of this figure.

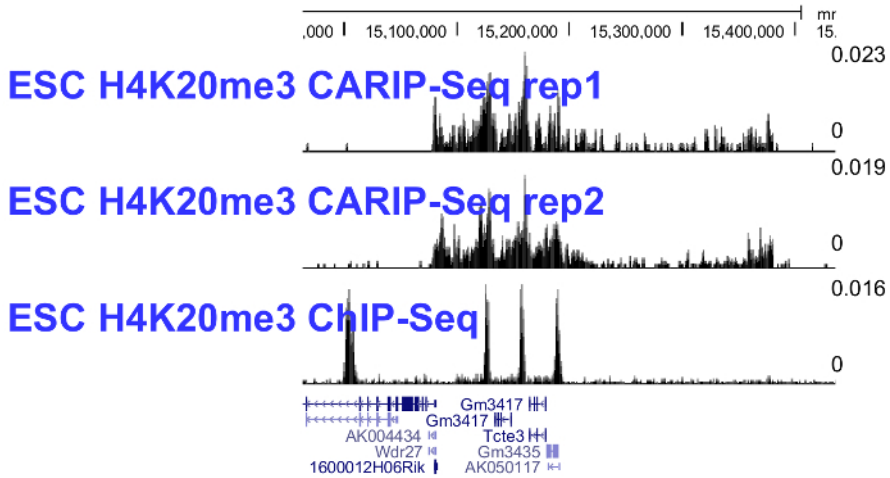


**Figure 4: Representative CARIP-Seq analysis of RNA associated with a histone modification and ChIP-Seq in ES cells.** UCSC browser views of H4K20me3 CARIP-Seq and H4K20me3 ChIP-Seq signals in ES cells (normalized tag density (RPBM), read per base per million reads). Please click here to view a larger version of this figure.

## Discussion

ChIP-Seq is a useful method to evaluate the location of global protein-DNA interactions (*e.g.*, transcription factors/histone modifying enzymes/ histone modifications and DNA) in ES cells, while the newly developed CARIP-Seq protocol is useful in interrogating genome-wide association

of RNAs with chromatin constituents. ChIP-Seq is a fundamental tool that is used to evaluate epigenetic landscapes of ES cells and other cell types. The quality of ChIP-Seq and CARIP-Seq libraries is largely dependent on ChIP-grade antibodies. The suitability of antibodies for ChIP-Seq can be empirically determined by performing ChIP-PCR, where a ≥ 3 to 5-fold enrichment at positive control regions relative to unoccupied regions should be sufficient to generate high-quality ChIP-Seq data[25]. Likewise, antibodies can be tested for their suitability for CARIP-Seq in a similar manner as described for ChIP-Seq with minor modifications. Following the RNA IP step in the CARIP protocol as described in the methods section above, reverse-transcription PCR should be performed to generate cDNA, which can be used as a template to perform Q-RT-PCR. It is recommended to evaluate the enrichment following ChIP or CARIP at several genomic regions. There are additional considerations to test when evaluating the quality of antibodies for ChIP or CARIP. For example, the stringency of buffers used for ChIP and CARIP may be optimized by altering the concentration of NaCl: a low NaCl concentration is suitable for highly-specific antibodies, while a high NaCl concentration may be helpful for antibodies with non-specific binding. Regarding the library preparation for next-generation sequencing, custom primers and indexes can also be used in lieu of commercially acquired oligonucleotides. However, it is important to note that including a phosphorothioate modification between two bases at the 3' end has been shown to prevent nuclease digestion[26]. Third-party RNA-Seq or ChIP-Seq library construction kits may also be used to generate libraries for sequencing of CARIP and ChIP samples, respectively. However, it is important to empirically test the kits to evaluate their efficacy in generating quality libraries for next-generation sequencing. Additional protocols may also be employed to perform ChIP-Seq library preparation, such as ChIPmentation[27]. However, adaptation of ChIPmentation to the ChIP-Seq protocol described here will require additional modifications and further optimization.

Here, we describe ChIP and CARIP protocols that have been optimized using moderate numbers of cells ($4 \times 10^6$ cells per IP). While the ChIP protocol can be performed using low to intermediate cell numbers ($10^5$ to $10^6$ cells), we have not performed CARIP-Seq using less than $2 \times 10^6$ cells. However, by empirically testing various fixation and sonication conditions, it is possible that this protocol may work successfully using a small number of cells.

In summary, ChIP-Seq and CARIP-Seq are useful for generating global maps of chromatin-associated proteins and RNAs in ES cells, respectively. While these protocols have been optimized for ES cells, they can be optimized to generate chromatin maps using a wide variety of mammalian cell lines and tissue types. These protocols may also be optimized to generate single-cell ChIP-Seq or CARIP-Seq libraries.

## Disclosures

The authors declare no conflict of interest.

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**AUTHORS' CONTRIBUTIONS:**

B.L.K. conceived of the CARIP-Seq method, designed and carried out the ChIP-Seq and CARIP-Seq experiments, analyzed the sequencing data, and drafted the manuscript. All authors have read and approved the final version of this manuscript.

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