Video Article

Chromatin Isolation by RNA Purification (ChIRP)

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Abstract

Long noncoding RNAs are key regulators of chromatin states for important biological processes such as dosage compensation, imprinting, and developmental gene expression [1,2,3,4,5,6,7]. The recent discovery of thousands of lncRNAs in association with specific chromatin modification complexes, such as Polycomb Repressive Complex 2 (PRC2) that mediates histone H3 lysine 27 trimethylation (H3K27me3), suggests broad roles for numerous lncRNAs in managing chromatin states in a gene-specific fashion [8,9]. While some lncRNAs are thought to work in cis on neighboring genes, other lncRNAs work in trans to regulate distantly located genes. For instance, *Drosophila* lncRNAs roX1 and roX2 bind numerous regions on the X chromosome of male cells, and are critical for dosage compensation [10,11]. However, the exact locations of their binding sites are not known at high resolution. Similarly, human lncRNA HOTAIR can affect PRC2 occupancy on hundreds of genes genome-wide [3,12,13], but how specificity is achieved is unclear. LncRNAs can also serve as modular scaffolds to recruit the assembly of multiple protein complexes. The classic trans-acting RNA scaffold is the TERC RNA that serves as the template and scaffold for the telomerase complex [14]; HOTAIR can also serve as a scaffold for PRC2 and a H3K4 demethylase complex [13].

Prior studies mapping RNA occupancy at chromatin have revealed substantial insights [15,16], but only at a single gene locus at a time. The occupancy sites of most lncRNAs are not known, and the roles of lncRNAs in chromatin regulation have been mostly inferred from the indirect effects of lncRNA perturbation. Just as chromatin immunoprecipitation followed by microarray or deep sequencing (ChIP-chip or ChIP-seq, respectively) has greatly improved our understanding of protein-DNA interactions on a genomic scale, here we illustrate a recently published strategy to map long RNA occupancy genome-wide at high resolution [17]. This method, Chromatin Isolation by RNA Purification (ChIRP) (**Figure 1**), is based on affinity capture of target lncRNA:chromatin complex by tiling antisense-oligos, which then generates a map of genomic binding sites at a resolution of several hundred bases with high sensitivity and low background. ChIRP is applicable to many lncRNAs because the design of affinity-probes is straightforward given the RNA sequence and requires no knowledge of the RNA's structure or functional domains.

Video Link

The video component of this article can be found at https://www.jove.com/video/3912/

Protocol

1. Probe Design

Design anti-sense DNA tiling probes for selective retrieval of RNA target by ChIRP.

1. Design anti-sense oligo probes using the online probe designer at singlemoleculefish.com [18].
2. Use these parameters: number of probes = 1 probe /100 bp of RNA length; 2) Target GC% = 45; 3) Oligonucleotide length = 20; 4) Spacing length = 60-80. Break RNA into segments if too long for the designer. Omit regions of repeats or extensive homology.
3. Order anti-sense DNA probes with BiotinTEG at 3-prime end.
4. Label probes according to their positions along the RNA. Separate them into two pools so that the "even" pool contains all probes numbering 2, 4, 6, etc. and the "odd" pool contains probes numbering 1, 3, 5, etc. Dilute pool of probes to 100 µM concentration and store at -20 °C.
5. All experiments are to be performed using both pools, which serve as internal controls for each other. Real RNA-dependent signal would be present from both pools, while probe-specific noises would be unique to each pool. This applies for both ChIRP-qPCR and ChIRP-seq.

2. Harvest Cells

Collect cells that will be used for ChIRP experiment.

1. Grow cells in tissue culture plates or flasks to confluency. Rinse with phosphate buffered saline (PBS) once and trypsinize. Quench trypsin with >2x volume of media, pipette up and down to dislodge cells and resuspend into single cell suspension. Transfer all media and resuspended cells into 50 ml Falcon tubes. 20 million cells are typically sufficient for one ChIRP sample.

2. Spin cells at 800RCF for 4 min. Aspirate media and resuspend 40 million cells in 40 ml of PBS, combine tubes if necessary. Spin cells at 800RCF for 4 min. Decant PBS, carefully aspirate on an angle the remaining liquid.

3. Cross-link Cells and Collect Cell Pellet

Crosslink collected cells with glutaraldehyde to preserve RNA-Chromatin interactions and prepare cell pellet.

1. Perform all steps at room temperature.
2. Prepare 1% glutaraldehyde in room temperature PBS. Prepare 10 ml per 10 million cells (0.4 mL 25% glutaraldehyde stock + 9.6 mL PBS). Glutaraldehyde must be used fresh.
3. Tap bottom of Falcon tubes to dislodge pellets. Resuspend cell pellet in 1% glutaraldehyde, starting with a small volume to avoid chunks, then top up to full volume. Invert to mix. Crosslink for 10min at room temperature on an end-to-end shaker or rotator.
4. Quench the cross-linking reaction with 1/10th volume of 1.25 M glycine at room temperature for 5 min.
5. Spin at 2000RCF for 5 min. Aspirate supernatant and wash pellet with 20 mL chilled PBS once, spinning at 2000RCF for 5 min.
6. Aspirate and resuspend the washed, cross-linked pellet with 1 ml chilled PBS per 20 million cells. Transfer each ml to an Eppendorf tube and spin at 2000RCF for 3 min at 4 C. Remove as much PBS as possible with pipette tip carefully.
7. Flash-freeze the cell pellets in liquid nitrogen and store at -80 °C indefinitely.

4. Cell Lysis

Lyse crosslinked cells to prepare cell lysate.

1. Thaw frozen cell pellets at room temperature. Tap hard to dislodge and mix the cell pellet. Spin down the pellet at 2000RCF for 3 min at 4 °C. Use a sharp 10 µl pipette tip to remove any remaining PBS.
2. On an electronic balance (accurate to 1mg) tare the mass of an empty Eppendorf tube (our tubes weigh 1.060 grams very consistently). Weigh each pellet and record its weight. A full 15 cm dish of crosslinked HeLa cells typically weighs 100 mg.
3. Supplement Lysis Buffer (10X the mass of pellet, e.g. 1 ml for 100mg) with fresh Protease Inhibitor, PMSF and Superase-in (see attached buffer list). Mix well.
4. Add 10X volume supplemented Lysis Buffer to each tube and resuspend the pellet. For small pellets <25 mg, resuspend in 250 µl supplemented lysis buffer. Suspension should be smooth. If not, divide suspension into 500 µl aliquots and use a motorized pellet mixer to break up clumps. Proceed immediately to sonication.

5. Sonication

Shear DNA by sonicating crosslinked cell lysates.

1. Sonicate cell lysate in Bioruptor in 15 ml Falcon tubes. Use <1.5 ml lysate in each tube, and for faster sonication, sonicate no more than two tubes at a time.
2. Sonicate in a 4 °C water bath at highest setting with 30 seconds ON, 45 seconds OFF pulse intervals. Check lysate every 30 min. Continue sonicating until the cell lysate is no longer turbid. This may take as little as 30 min and as many as 4 hrs. The number of tubes, the sample volume, the bath temperature, and the period of sonication time will affect how long the process takes. Tubes will likely sonicate at different rates, so pool them together every 30 min and redistribute into original tubes to ensure homogeneity. Note: glutaraldehyde-crosslinked cells take significantly longer to sonicate than formaldehyde equivalents.
3. When lysate turns clear, transfer 5 µL lysate to a fresh Eppendorf tube. Add 90 µL DNA Protease K (PK) Buffer (see buffer list) and 5 µL PK. Vortex to mix and spin down briefly. Incubate for 45 min at 50 °C.
4. Extract DNA with Qiagen PCR purification kit. Elute DNA in 30 µL Qiagen Elution Buffer (EB) and check DNA size on 1% agarose gel. If bulk of the DNA smear is 100-500 bp, sonication is complete. If not, continue to sonicate.
5. Centrifuge sonicated samples at 16100RCF for 10 min at 4 °C. Combine supernatants, aliquot into 1 mL samples and flash-freeze in liquid nitrogen. Store at -80 °C.

6. ChIRP

Hybridize biotinylated DNA probes to RNA and isolate bound chromatin.

1. Thaw tubes of chromatin at room temperature.
2. Prepare Hybridization Buffer (see buffer list, prepare 2 ml per ml of chromatin). Vortex to mix.
3. For a typical ChIRP sample using 1 ml of lysate, remove 10 µL for RNA INPUT and 10 µL for DNA INPUT and place in Eppendorf tubes. Keep on ice till further use.
4. Transfer 1 mL chromatin to 15 mL Falcon tube. Add 2 mL Hybridization Buffer to each tube. For total volume <1.5 ml, use Eppendorf tubes.
5. Thaw probes at room temperature. Nanodrop probes to check amount if you haven't used it in a long time (100 µM probes should spec ~500-600 ng/µl using single strand DNA setting). Add appropriate volume of probes to specific tubes (100 pmol probe per 1 mL chromatin, 1 µL of 100 pmol/µL probe per 1 mL chromatin). Mix well. Incubate at 37 °C for 4 hrs with shaking.
6. With 20 min remaining for hybridization, prepare the C-1 magnetic beads (stored at 4 °C). Use 100 µL per 100 pmol of probes. Wash with 1 mL unsupplemented Lysis Buffer three times, using the DynaMag-2 magnet strip to separate beads from buffer.
7. Resuspend beads in original volume of Lysis Buffer; supplement with fresh PMSF, P.I and Superase-in. After 4 hr hybridization reaction is complete, add 100 µL beads to each tube. Mix well. Incubate at 37 °C for 30 min with shaking.
8. Prepare Wash Buffer (5 mL per sample). Vortex to mix. Pre-warm to 37 °C. Add PMSF before use.

9. Wash beads with 1 mL wash buffer five times. On the first wash, use DynaMag-15 magnetic strip to separate beads, decant, and resuspend in 1 mL wash buffer. Transfer volume to 1.5 mL Eppendorf tube. Incubate at 37 °C with shaking for 5 min.
10. On subsequent washes, spin down each tube on a minicentrifuge, set sample on DynaMag-2 magnetic strip for 1 min. Decant sample, wipe any drips with a Kimwipe, resuspend in 1 mL wash buffer. Incubate at 37 °C with shaking for 5 min. Repeat for five total washes.
11. At last wash, resuspend the beads well. Remove 100 µL and set aside for RNA isolation. Reserve 900 µL for DNA fraction. Place all tubes on DynaMag-2 magnetic strip and remove wash buffer. Spin all tubes down briefly; place them on magnet strip. Remove the last bit of wash buffer completely with a sharp 10 µl pipette tip.

7. RNA Isolation

Extract RNA fraction from ChIRP samples to quantitate by qRT-PCR.

1. Take 100 µL bead samples and 10 µL RNA INPUT sample. Add 85 µL RNA PK Buffer pH 7.0 to RNA INPUT. Resuspend beads in 95 µL RNA PK Buffer pH 7.0. Add 5 µL Proteinease K and incubate at 50 °C for 45 min with end-to-end shaking.
2. Briefly spin down all tubes and boil samples for 10 min on heat block at 95 °C.
3. Chill samples on ice, add 500 µL TRIzol, vortex vigorously for 10 sec. Incubate at room temperature for 10 min. Store at -80 °C or proceed to step 4.
4. Add 100 µL chloroform to TRIzol treated samples. Vortex vigorously for 10 sec. Spin at 16100RCF on a benchtop centrifuge for 15 min at 4 °C.
5. Remove ~400 µL aqueous supernatant, avoiding organic and interface.
6. Add 600 µL (1.5 volume) 100% ethanol and mix well. Spin sample through MIRNeasy mini columns. Wash 1x with RWT (MIRNeasy mini kit), 2x with RPE per manufacturer's protocol. Elute with 30 µL nuclease-free H_2O (nfH_2O).
7. Treat the RNA eluate with DNA-*free* per manufacturer's protocol. After the reaction is complete, heat the sample for 15 minutes at 65 °C to completely inactivate any remaining DNase.
8. Use 1 µL RNA isolate per well for qRT-PCR analysis to confirm lncRNA retrieval. GAPDH is often used as a negative control.

8. DNA Isolation

Extract DNA fraction from ChIRP samples to identify by sequencing or quantitate by qPCR.

1. Prepare DNA Elution Buffer (see buffer list), 150 µl per sample, including DNA INPUT.
2. Add 1 0µL RNase A (10 mg/mL) and 10 µL RNase H (10 U/µl) per ml of DNA Elution Buffer, and vortex to mix.
3. Resuspend each sample of beads in 150 µL of DNA Elution Buffer with RNases. (Resuspend DNA INPUT in 140 µL) Incubate at 37 °C for 30 min with shaking.
4. Separate beads and supernatant on DynaMag-2 magnetic strip. Remove supernatant and add to labeled tubes.
5. Prepare a second aliquot of DNA Elution Buffer with 10 µL RNase A (10 mg/mL) and RNaseH (10 U/µL) exactly as done in 8.2). Add 150 µL to each sample (including DNA INPUT), incubate, and remove supernatant. Collect all supernatant (should be ~300 µL).
6. Add 15 µL PK to each sample. Incubate at 50 °C for 45 min with shaking.
7. Pre-spin down yellow phase-lock gel tubes (5PRIME). Transfer DNA samples to phase-lock gel tubes, and add 300 µL PhOH:Chloroform:Isoamyl per sample. Shake vigorously for 10 min, and spin down on a benchtop centrifuge at 16100RCF for 5 min at 4 °C. Take aqueous from the top (~300 µL). Add 3 µL GlycoBlue, 30 µL NaOAc, and 900 µL 100% EtOH. Mix well and store at -20 °C overnight.
8. Spin samples at 16100RCF for 30 min at 4 °C.
9. Decant supernatant carefully. Add 1 mL 70% EtOH and vortex to mix. Spin at 16100RCF for 5 min. Remove supernatant by pipette. Air dry for 1min. Resuspend in 30 µL EB.
10. DNA samples are ready for analysis by qPCR or preparation of high-throughput sequencing libraries per Illumina protocol.

10. Representative Results

Figure 1 depicts the ChIRP workflow. A successful ChIRP experiment typically enriches target RNA significantly over non-specific RNAs. **Figure 2** shows enrichment of human telomerase RNA (TERC) from HeLa cells over GAPDH, an abundant cellular RNA that serves as a negative control. Majority of TERC RNAs (~88%) present in the cell were pulled down by performing ChIRP, whereas only 0.46% of GAPDH RNA was retrieved, demonstrating an enrichment factor of ~200 fold. Nonspecific probes such as probes targeting LacZ RNA, which is not expressed in mammalian cells (**Figure 2**), can be used as additional negative controls.

DNA regions expected to bind the target lncRNA are typically enriched over negative regions when measured by qPCR. **Figure 3** shows qPCR validation of four HOTAIR-bound sites in primary human foreskin fibroblasts that we determined by performing ChIRP-seq in the same cell line, while TERC and GAPDH DNA sites serve as negative control regions. Both "even" and "odd" probe sets yielded comparable enrichment of expected HOTAIR-bound sites over negative regions, a hallmark of true lncRNA-binding sites.

High-throughput sequencing of ChIRP enriched DNA yields a global map of lncRNA-binding sites. The *Drosophila* lncRNA roX2 is known to interact with the X-chromosome in a manner that is required for dosage compensation. **Figure 4** shows roX2 binding profile over a section of the X chromosome. Both "even" and "odd" samples have been sequenced and their unique noises have been eliminated to produce a track of overlapping signals. Each "peak" here indicates a strong site of roX2 binding. The complete track and list of roX2 target genes have been described in Chu *et al.* 2011 [17].

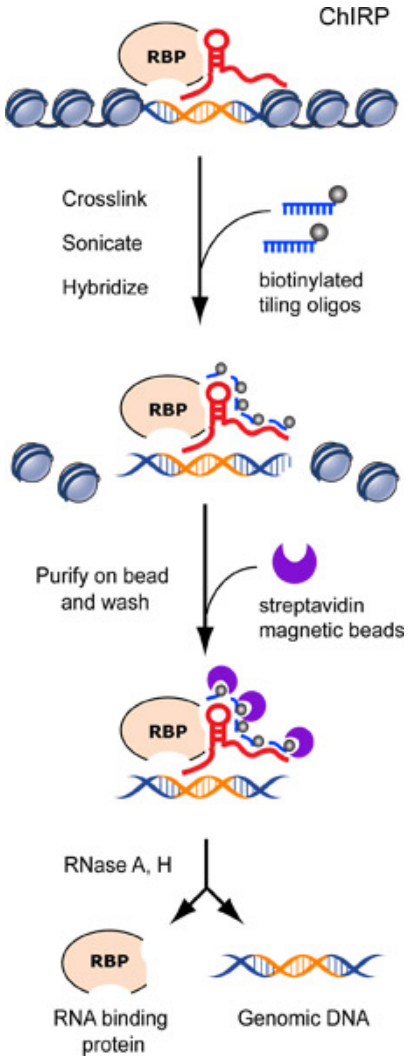

Figure 1. Flow chart of the ChIRP procedure. Chromatin is crosslinked to lncRNA:protein adducts *in vivo.* Biotinylated tiling probes are hybridized to target lncRNA, and chromatin complexes are purified using magnetic streptavidin beads, followed by stringent washes. We elute lncRNA bound DNA or proteins with a cocktail of Rnase A and H. A putative lncRNA binding sequence is schematized in orange. Previously published in Chu *et al.* 2011.[17]

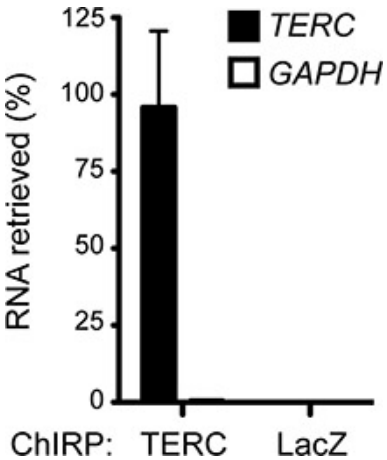

Figure 2. ChIRP enriches for human TERC RNA. TERC-asDNA probes retrieve ~88% of cellular TERC RNA and undetectable GAPDH. LacZ-asDNA probes are used as negative controls and retrieve neither RNAs. Mean + s.d. are shown. Previously published in Chu *et al.* 2011.[17]

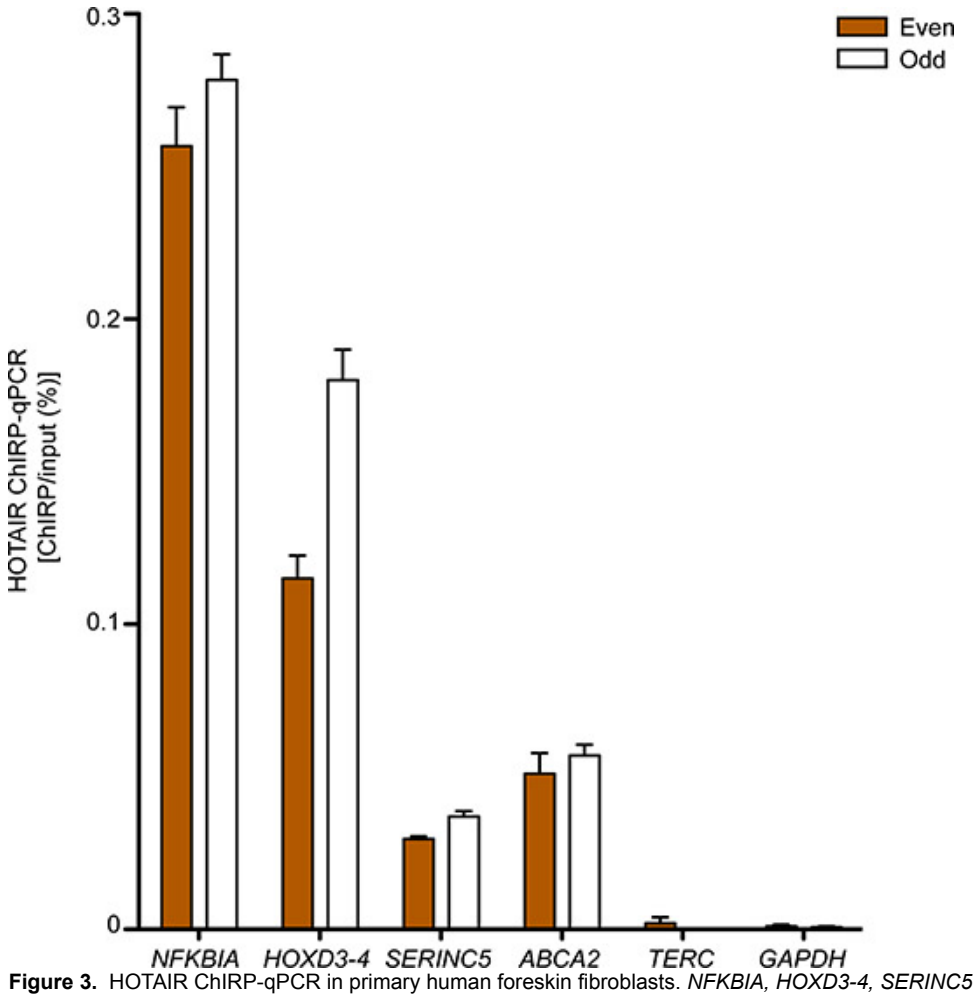

Figure 3. HOTAIR ChIRP-qPCR in primary human foreskin fibroblasts. *NFKBIA, HOXD3-4, SERINC5 and ABCA2* are regions that interact with HOTAIR. *TERC* and *GAPDH* served as negative controls. Mean + s.d. are shown. Previously published in Chu *et al.* 2011.[17]

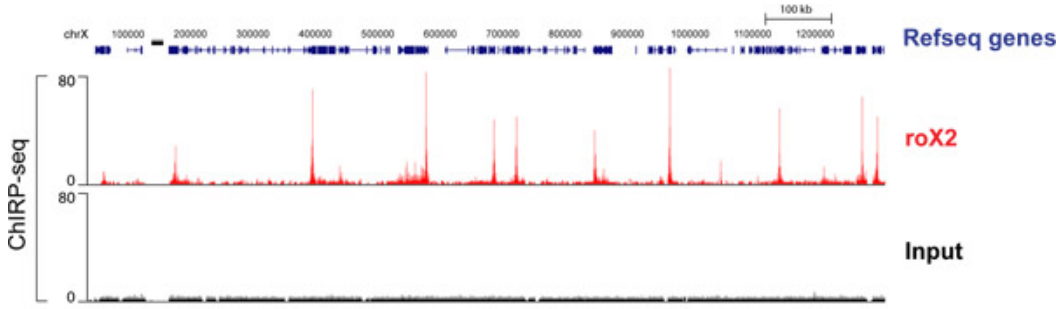

Figure 4. ChIRP-seq data of roX2 RNA in SI2 *Drosophila* cells. "Even" and "odd" were sequenced separately; their data merge to reflect only common peaks in both. The merged track is shown. Previously published in Chu *et al.* 2011.[17]

Discussion

Here we described ChIRP-seq, a method of mapping *in vivo* lncRNA binding sites genome-wide. The key parameters for success are the split pools of tiling oligonucleotide probes and glutaraldehyde crosslinking. The design of affinity-probes is straightforward given the RNA sequence and requires no prior knowledge of the RNA's structure or functional domains. Our success with roX2, TERC, and HOTAIR - three rather different RNAs in two species - suggests that ChIRP-seq is likely generalizable to many lncRNAs. As with all experiments, care and proper controls are required to interpret the results. Different lncRNA may require titration of conditions, and judicious change of conditions, such as selection of different affinity probes or crosslinkers, may highlight different aspects of RNA-chromatin interactions. Like ChIP-seq, not all binding events are necessarily functional, and additional studies are required to ascertain the biological consequences of RNA occupancy on chromatin. Nonetheless, we foresee many interesting applications of this technology for researchers of other chromatin-associated lncRNAs, which now number in the thousands[8,9]. Just as ChIP-seq has opened the door for genome-wide explorations of DNA-protein interactions, ChIRP-seq studies of the "RNA interactome" may reveal many new avenues of biology.

Disclosures

C. Chu and H.Y. Chang are named as inventors on a patent application based on this method.

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