Magna ChIRP™ RNA Interactome Kits

Analysis and Mapping of Chromatin: Noncoding RNA Interactions

Magna ChIRP RNA Interactome Kit (Catalog No. 17-10494)
EZ- Magna ChIRP RNA Interactome Kit (Catalog No. 17-10495)

12 reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Gene regulation plays a critical role in complex cellular processes such as development, differentiation, and cellular response to environmental changes. While the regulation of gene expression by transcription factors and epigenetic influences has been well studied over time, pervasive genomic transcription and the role of non-coding RNAs in this process is a rapidly evolving field that remains to be thoroughly explored.

Chromatin is typically thought of as a complex of DNA, histones, and non-histone proteins. The RNA component of chromatin was considered to be composed of mRNAs or traditional snRNAs that would transiently associate with chromatin during transcription. However, mounting evidence suggests that various classes of non-coding RNAs (e.g. long non-coding RNAs, enhancer RNAs and even miRNAs) associate with chromatin and serve regulatory functions possibly through sequence-specific hybridization and/or through structural and spatial mechanisms. Approaches that allow one to identify and characterize interactions between RNA molecules (both coding and non-coding), proteins and DNA are needed to better characterize these regulatory mechanisms.

Historically, chromatin immunoprecipitation (ChIP) has been used to interrogate association of proteins with genomic DNA sequences. Given that chromatin contains not only DNA and DNA binding proteins, but also RNA binding proteins and RNA, new methods have been developed to map the association of these RNA molecules to distinct regions of the genome. Antibody-based methods such as CLIP and nuclear RIP (see Magna Nuclear RIP user manual cat# 17-10520 and 17-10522 for details) allow isolation and identification of RNA molecules associated with chromatin. Recently, other methods have been established that allow researchers to examine interactions among proteins or genomic DNA sequences by using probe-based hybridization to target RNA molecules in chromatin. One such method, designated ChIRP[1] (Chromatin Isolation by RNA Purification), allows recovery of specific segments of DNA and proteins using chromatin-associated RNA as a target. This unique approach allows discovery of the sites of interaction of chromatin-associated RNAs (e.g. lncRNAs) with genomic DNA sequences, as well as proteomic analysis of protein components of the lncRNA regulatory complexes.

Overview of the Magna ChIRP Method

The Magna ChIRP RNA Interactome Kit uses the ChIRP method to enable identification and analysis of regions of genomic DNA that interact with a particular chromatin-associated RNA. The novel probe-based capture strategy employed in the Magna ChIRP Kit allows unbiased discovery of RNA-associated DNA sequences and proteins. To perform ChIRP multiple biotinylated oligonucleotide probes complementary to the RNA of interest are used (see figure 1). To help eliminate non-specific signals two different pools of probes are used (even and odd probe sets). These probe sets are combined with chromatin and hybridized to the chromatin-associated RNA. Complexes containing biotinylated-probes bound to the chromatin-associated RNA are then isolated using streptavidin magnetic beads (see figure 1). DNA can then be recovered and analyzed by quantitative PCR or next generation sequencing (ChIRP-seq). Alternatively, RNA may also be isolated from an aliquot of the recovered chromatin to detect other RNA molecules that may be associated with the RNA of interest.

Overview of Magna ChIRP Kit Workflow

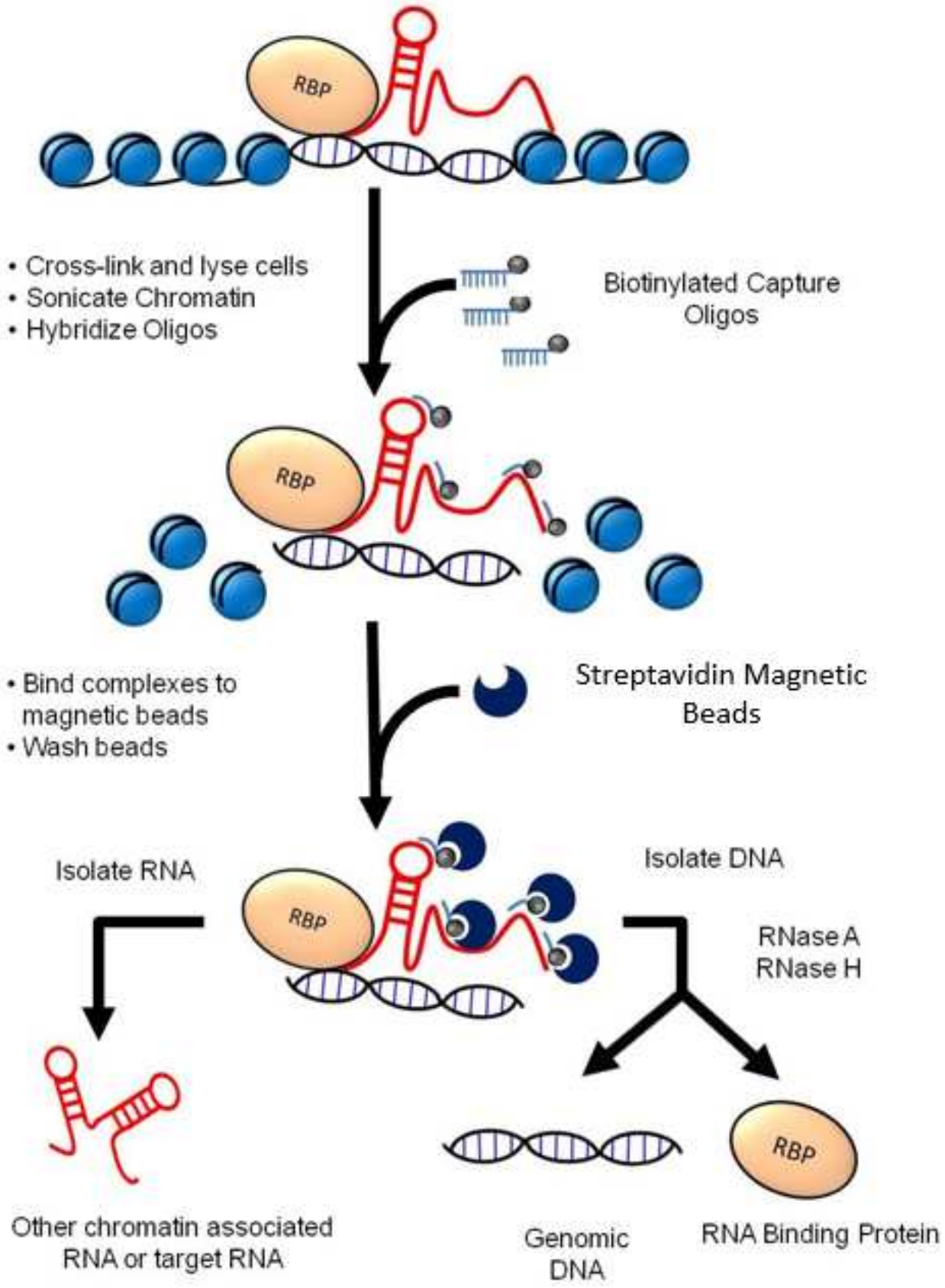


Figure 1. The Magna ChIRP™ Process

Materials Provided (Kit Configurations)

The ChIRP kit provides sufficient reagents for 7 individual chromatin preparations using 2 x 10^7cells and 12 ChIRP reactions. The EZ- ChIRP kit contains all the reagents included in the ChIRP kit plus positive control ChIRP Probes as well as positive and negative DNA and RNA detection primer sets for qPCR and qRT-PCR analysis, respectively. Please refer to the table below for details on kit components.

CHIRP01 (Component box of all ChIRP Kits) Store at 4 °C		
Component	**Part #**	**Quantity**
Streptavidin Magnetic Beads	CS220044	1.5 mL
10X Glycine	CS207370	27 mL
Lysis Buffer	CS216587	60 mL
Hybridization Buffer (without 15% formamide)	CS216571	24 mL
Wash Buffer	CS216569	60 mL
Proteinase K Buffer (for DNA)	CS216568	0.9 mL
Proteinase K Buffer (for RNA)	CS216567	1.65 mL
DNA Elution Buffer	CS216566	5.4 mL
0.5M EDTA	CS203175	0.5 mL

CHIRP02 (Component box of all ChIRP Kits) Store at -20℃		
RNase Inhibitor	CS216144	200 µL
Protease Inhibitor Cocktail III, Animal Free **Contains DMSO	535140-1ML	1 mL
Proteinase K Solution, 600mAU/mL	CS207286	2 x 200 µL
DNase I RNase free	CS216565	18 µL
DNase I Reaction Buffer	CS216135	75 µL
RNase A (20 mg/mL)	20-297	60 µL
RNase H (10U/ µL)	CS216564	54 µL
Magna ChIRP™ Negative Control Probe Set (LacZ) 50 µM	CS216572	12 µL

CHIRP03 (Component box of 17-10495 only) Store at -20℃)		
Magna ChIRP™ TERC lncRNA Probe Set (Even) 50 µM	CS216575	12 µL
Magna ChIRP™ TERC lncRNA Probe Set (Odd) 50 µM	CS216563	12 µL
RNA Positive Control Primers (TERC Gene) F: CGC TGT TTT TCT CGC TGA CT R: GCT CTA GAA TGA ACG GTG GAA	CS216598	75 µL
RNA Negative Control Primers (GAPDH Gene) F: GTC GGA GTC AAC GGA TTT G R: TGG GTG GAA TCA TAT TGG AA	CS216610	75 µL
Magna ChIRP™ Primers, WNT-1 precursor F: AGG GCT GGA ATT TCA AAG GT R: TTC TCC TCA GGA TGT ACC CG	CS216609	75 µL
ChIP Primers, GAPDH coding D2 F: GGC TCC CAC CTT TCT CAT CC R: GGC CAT CCA CAG TCT GG	CS207323	75 µL

Materials Required But Not Supplied

Reagents

- Cells, stimulated or treated as needed for the experimental system
- PBS (RNase free)
  (e.g. Fisher, Cat. # BP2438-4)
- Glutaraldehyde solution (e.g. SIGMA, Cat. # G5882-10X10 mL)
- Formamide (EMD Millipore Cat. # 4610-100ML)
- Anti-sense oligo probes
- TRIzol® Reagent (Life Technologies Cat. #15596-018)
- Chloroform (EMD Millipore, Cat. #3150)
- 100% Ethanol (molecular biology grade)
- Phenol: Chloroform: Isoamyl alcohol, 25:24:1, TE Buffered Saturated pH 8.0 (EMD Millipore Cat. # 6805)
- Precipitation Carrier
  - Pellet Paint®NF Co-Precipitant (125 reactions, EMD Millipore Cat. # 70748-3) or RNase-free glycogen
  - If sample are intended for Next Gen Sequence library preparation, use Linear Acrylamide (5mg/mL, Life Technologies Cat. # AM9520)
- 3M Sodium Acetate (EMD Cat. # 567422-100ML)
- Nuclease Free Water
- 10 mM Tris- HCl, pH 8.5
- PCR purification kit (QIAGEN, Cat. # 28106)
- miRNeasy® Mini kit (QIAGEN, Cat. # 217004) or other RNA purification column kit
- Phase Lock Gel Heavy (5 PRIME, Cat. # 2302810)
- Conical tube (15 mL and 50 mL)
- Nuclease-free Microcentrifuge tubes, 1.5 mL, 2.0 mL
- PCR plate, 0.2 mL
- Liquid nitrogen (Optional)

Reagents for qRT-PCR Analysis

- One-Step RT-PCR Reagent (e.g. Bio-Rad iTaq™ Universal SYBR® Green One-Step Kit Cat. # 172-5150)

Reagents for qPCR Analysis

qPCR Reagent (e.g. Bio-Rad iQ™ SYBR® Green Supermix Cat. # 170-8880)

Reagents for ChIRP-Seq Library Construction

- Sequence library preparation kit
  - PureGenome™ Low Input NGS Library Construction Kit (Cat. # 17-10492) or other low input DNA next generation sequencing library construction kit
  -

Equipment

- Sonicator, Water bath sonicator such as Q800R Sonicator (Qsonica) is recommended
- Hybridization Oven
- Magnetic Separation Rack
  - Magna GrIP™ Rack (8 well, Millipore Cat. # 20-400) or PureProteome™ Magnetic Stand, (Millipore Cat. # LSKMAGS08 and LSKMAGS15)
- Vacuum Aspirator
- Vortex mixer
- Rotating wheel/platform
- Centrifuge for cell culture
- Microcentrifuge
- Ultra low temperature freezer (below -80℃)
- Thermomixer® (60℃ capable)
- Variable temperature water bath or incubator
- Rotating microtube mixer
- Timer
- Pipette (2 mL, 5 mL, 10 mL, 25 mL)
- Variable volume (5-1000 µL) pipettes
- Nuclease-free filter pipette tips
- Cell scraper
- Real-time PCR thermal cycler
- NanoDrop™ or spectrophotometer

Important Notes Before Starting

Please read through the entire protocol and carefully plan your work before starting. The ChIRP method requires multiple steps and can be done over a two-day period or over multiple days. There are several stopping points to allow the method to be carried out over multiple days. The approximate time required for each step and potential stopping points are provided in the tables below.

Cross-linking, Lysis, Hybridization, and Recovery of Complexes

Protocol Step	Time Required	Stopping Points and Protocol Notes
Cross Link Cells	~2 hours	Can snap freeze cell pellet in liquid nitrogen and store at -80°C
Cell Lysis and Sonication	~8 hours	Sonicated lysate may be stored for up to 3 months at -80℃
Magnetic Bead Based Isolation of Chromatin with Biotinylated Probes	~5 hours	Continue to DNA or RNA recovery and analysis step

Recovery and Analysis of DNA

Protocol Step	Time Required	Stopping Points and Protocol Notes
DNA Isolation	~2.5 hours to Overnight	DNA can be stored for several weeks in ethanol at -20℃
DNA Analysis	~3 hours for qPCR	Note that analysis can also be performed by DNA-seq

Recovery and Analysis of RNA

Protocol Step	Time Required	Stopping Points and Protocol Notes
RNA Isolation	~2.5 hours	None
DNA Digestion	~0.5 hours	DNA can be stored for several weeks in ethanol at -20℃
RNA Analysis	~4 hour for RT-qPCR	Note that analysis can also be performed by RNA-seq

RNase control

Throughout this method, all standard precautions should be taken to minimize RNase contamination. Gloves should be worn at all steps of the procedure. All instruments, glassware and plastics that touch cells or cell lysates should be certified nuclease-free or should be pretreated using DEPC or other RNase inactivation reagents according to established protocols for working with RNA. RNase inhibitor (Part# CS216144) is included as a component in this kit. All solutions utilized that are not kit components should be certified DNase-free and RNase-free from the manufacturer wherever possible.

Detailed Protocol

If you are new to the Magna ChIRP method, please read and understand this entire protocol before starting. This version of the protocol contains important details and helpful tips to facilitate a successful result. For advanced users, a summary protocol is presented starting on page 20.

Capture Probe Design

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

1. Design anti-sense oligo probes using following parameters.
    a. number of probes = 1 probe / 100 nucleotides of RNA
    b. Target GC% = 45
    c. Oligonucleotide length = 20
    d. Spacing length = 60-80
        - *Online probe design programs such as www.singlemoleculefish.com can be used*
        - *Divide the RNA sequence into segments if too long for the probe design program of choice.*
        - *Omit regions of repeats or those with extensive homology.*
2. Label probes (1, 2, 3, 4, 5…) according to their positions along the target RNA. Separate biotinylated probes into two groups (even and odd) so that "even" group contains all probes numbering 2, 4, 6, etc. and the "odd" group contains probes numbering 1, 3, 5, etc.

Table 1. Even and Odd Pools of Probes

Probe No.	1	2	3	4	5	6	7	8	-----	-----
even pool		x		X		x		x		
odd pool	x		x		x		x			

3. Ensure no oligo sequences from the even pool can share homology longer than 13 bp with those of the odd pool.
    - *This is especially important for CHIRP-seq analysis. If there are two oligos in opposite pools that share (approximately) >13bp of homology (sense OR antisense orientations), they can both hybridize to genomic DNA at the same locus; when sequencing data is merged, this generates peak artifacts at sites in the genome matching the shared homologous sequences.*
    - *Online motif discovery programs such as MEME (http://meme.nbcr.net/meme/) can be used.*
    - *Load in all of the probe sequences, allow searching for forward and reverse-complement sequences, and look for any significant overlap between oligos in opposite pools.*
4. Synthesize anti-sense DNA probes with BiotinTEG at 3-prime end.
5. Prepare all probes at the same concentration. (50-100 µM)
6. Combine equivalent volumes of probes within the same group at identical concentrations and further dilute if necessary to **50 µM total oligo concentration**. Make several aliquots of each set of pooled probes to avoid multiple freeze-thaw cycles. Store the aliquots of pooled probes at -20 ℃. Probe pools require 2 µL per reaction.
    - *All experiments are performed using both pools, which serve as internal controls for each other. Real RNA-dependent signal would be present in both pools, while non-specific hybridization signals may be unique to each pool. This applies to both ChIRP-qPCR and ChIRP-seq.*
    - *Absolute expression levels of the target RNAs are important for the success of the experiment. Using qPCR, target RNAs should be detected at a Ct value below 23 using 100 ng of total RNA.*

A. Planning Lysate Requirements for ChIRP Experiments

- Calculate the number of desired ChIRP reactions. For each RNA target of interest use one even and odd probe pool and a single negative control probe pool. Magna ChIRP Kits (Cat. # 17-10494 and 17-10495) include a negative control pool (Magna ChIRP Negative Control Probe Set (LacZ) 50 µM; Part # CS216572). If you are using the EZ-Magna ChIRP kit (Cat. # 17-10495), a set of positive control probes are included (Magna ChIRP TERC lncRNA Probe Set (Even) 50 µM; Part # CS216575 and Magna ChIRP TERC lncRNA Probe Set (Odd) 50 µM; Part # CS216563). Both probe sets are also available separately. Please refer to the Related Products section on page 31 for ordering information.
- Typically one ChIRP reaction (i.e. one chromatin purification reaction using one probe pool) requires 1 mL of chromatin from ~1.0 x 10^7 cells (one 15 cm plate)**.** The calculation for the volume of Lysis Buffer required for a ChIRP experiment is based on the weight of the **cell pellet** harvested. This weight may vary depending on the type of cells utilized. Examples of the number of HeLa cells obtained from various cell culture vessels and volume of Lysis Buffer required are shown below (Table 2). In some cases the number of cells or amount of chromatin required may need to be optimized empirically. However, it is recommended that the amount of chromatin suggested in the protocol below be used for initial experiments. Once an initial positive ChIRP with a target RNA has been demonstrated, the amount of chromatin per ChIRP reaction may be reduced or further optimized as necessary.
- The total number of the cells or total amount of chromatin used per ChIRP reaction may be optimized depending on the abundance of the RNA target and DNA recovered as well as the method of detection.

Table 2. Approximate volumes of lysis buffer per cell culture vessel (HeLa cells)

Type of vessel	Surface Area (cm^2)	Cell Number	Volume of Lysis Buffer (µL)
T-75	75	~0.5 x 10^7	500
T-225	225	~1.3 x 10^7	1300
10 cm plate	78.5	~0.5 x 10^7	500
15 cm plate	176.6	~1.0 x 10^7	1000

B. In Vivo Cross linking of RNA/DNA and Protein (Chromatin)

1. Prepare cells. Stimulate or treat, if necessary, adherent mammalian cells at ~80 to 90% confluence in two 15 cm culture plates. Each plate will contain 20 mL of growth media. Include one extra plate of cells to be used solely for the estimation of cell number. If using suspension cells stimulate or treat 2 x 10^7 cells in 40 mL volume.
   - *For HeLa cells, two 15 cm plates is approximately 2 x 10^7 cells. This typically generates a preparation of chromatin sufficient for two ChIRP reactions (even and odd).*
   - *Cell numbers can be adjusted according to the performance of the probe of interest to optimize highest signal-to-noise ratio relative to negative control (LacZ probes or negative RNA control). This protocol is written using 1 X 10^7 cells per ChIRP reaction to ensure optimal performance when using the control probe. Note that even and odd probe pools are utilized for each ChIRP target so 2 x10^7 cells are required for each RNA target (1 x10^7 cells for the even probe pool and 1 x10^7 cells for the odd probe pool). The negative control probe pool requires an additional 1 X 10^7 cells.*

- *The volume of buffers supplied in the kit is sufficient to generate chromatin from up to fourteen 15 cm plates of cultured cells.*
- *Chromatin can be isolated from cell culture vessels other than those shown in Table 2 with slight modifications to the protocol.*

2. Trypsinize and Harvest (If using suspension cells go to step 3)
    a. Warm up PBS, 0.25% trypsin-EDTA, complete culture media, and fresh 25% glutaraldehyde stock to room temperature. Prepare 22 mL of chilled PBS per 2 x 10^7 cells for Steps 12 and 15.
        - *Use fresh high quality (molecular biology grade) glutaraldehyde each time. Do not use previously opened glutaraldehyde.*
    b. Aspirate media, rinse with 10 mL PBS and trypsinize with 5 ml of 0.25% Trypsin-EDTA for each 15 cm plate.
    c. Incubate the dish in a cell culture incubator (37℃) for 2-3 minutes. Monitor the cell morphology under a light microscope. When cells start appearing rounded, gently tap the flask to detach the cells completely from the surface. If cells do not detach after gentle tapping, incubate an additional 2-3 min in 37℃ inc ubator to completely detach cells.
    d. Quench trypsin by adding 10 mL of fresh complete culture media to each plate, pipette up and down to dislodge cells and break cell clumps. Transfer all media and resuspended cells in to 50 ml conical tubes.
3. Centrifuge at 800 x g for 5 minutes to pellet cells. Aspirate supernatant and resuspend 2 x 10^7 cells in 20 mL PBS. Combine tubes if necessary (maximum 4 x 10^7 cells or 40 mL for each 50 mL conical tube).
4. Centrifuge at 800 x g for 5 minutes to pellet cells.
5. Aspirate supernatant.
6. Resuspend the cell pellet in 1% glutaraldehyde PBS.
    a. Prepare 20 mL of 1% glutaraldehyde PBS per 2 x 10^7 cells at room temperature (dilute 0.8 mL of 25% glutaraldehyde stock with 19.2 mL of PBS)
    b. Tap bottom of the 50 mL conical tube to dislodge cell pellets. Resuspend cells in 20 mL of 1% glutaraldehyde PBS per 2 x 10^7 cells by inverting the tube several times to mix.
        - *Pipette up and down with a 10 mL pipette to suspend cell pellet completely.*
7. Incubate at room temperature (18-25ºC) for 10 minutes on an end-to-end shaker or rotator.
8. Add 2 mL of 10X glycine (Part # CS207370) to each 20 mL of 1 % glutaraldehyde PBS to quench excess glutaraldehyde. Invert the tube several times to mix.
9. Incubate at room temperature (18-25ºC) for 5 minutes on an end-to end shaker or rotator.
10. Centrifuge the cells at 2000 x g for 5 minutes.
11. Aspirate supernatant, removing as much medium as possible. Do not to disturb the cell pellet.
12. Resuspend the cells with 20 mL of cold PBS prepared in step 2 per 2 x 10^7 cells to wash.
13. Centrifuge the cells at 2000 x g for 5 minutes.
14. Carefully aspirate supernatant without disturbing cell pellet.
15. Resuspend the washed, cross-linked cell pellet with 2.0 ml of cold PBS per 2 x 10^7 cells.
16. Transfer each ml of cells in PBS to a 1.5 mL microcentrifuge tube.

17. Centrifuge the cells at 2000 x g for 3 minutes at 4º C. Carefully remove supernatant to avoid aspiration of cells with pipette tip
18. Cell pellet can be snap-frozen in liquid nitrogen and stored at -80° C at this point, or continue on to cell lysis and sonication.

C. Cell Lysis

If optimal conditions for sonication have already been determined, proceed with Section C. Otherwise see Appendix A for guidelines on optimization of sonication conditions.

1. If necessary, thaw cell pellets that have been stored at -80℃ on ice.
2. Tap hard to dislodge and mix cell pellet. Centrifuge cell pellet at 2000 x g for 3 minutes at 4℃.
3. Use a sharp 10-20 µL pipette tip to remove any remaining PBS.
4. Weigh each cell pellet.
   a. On an electronic balance (accurate to 1 mg) tare the mass of an empty microcentrifuge tube (the tubes usually weigh around 1.0 grams).
   b. Weigh each pellet and record its weight. A full 15 cm dish of cross-linked HeLa cells typically weighs 100 mg.
5. Prepare 1.0 mL of Complete Lysis Buffer for each 100 mg cell pellet by adding 5 µL of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML) and 5 µL of RNase inhibitor (Part # CS216144) to the Lysis Buffer (Part # CS216587). Mix well.
   - *Typical ChIRP experiment (even and odd) requires 2.0 mL of complete lysis buffer and total 200 mg of cell pellet. A negative control (LacZ) reaction requires additional 1.0 mL of complete lysis buffer and total 100 mg of cell pellet.*
6. Resuspend in 1.0 mL of complete Lysis Buffer per 100 mg cell pellet. Suspension should be smooth.
   - *It is recommended that cell concentration be at or less than 100 mg/mL, as the ratio of lysis buffer to cell density is important for reliable cell lysis. For small pellets <25 mg, resuspend in 250 µL supplemented lysis buffer.*
7. Proceed to immediately to Section D, Sonication to Shear DNA.

D. Sonication to Shear DNA

Important: Optimal conditions need to be determined to shear cross-linked DNA to ~100-500 base pairs in length. See Appendix A for a typical protocol. Once shearing conditions have been optimized, proceed with the steps below. The protocol presented below describes the use of a water bath sonicator (Q800R Sonicator, Qsonica) and is provided as an example only.

1. Sonicate the cell lysate.
   a. Pool the all cell lysate from Section C, Step 6 in a 15 mL conical tube. If desired, save 5 µL of the lysate for microfluidic electrophoresis (e.g. Agilent Bioanalyzer) or agarose gel analysis of unsheared chromatin along with the corresponding sheared chromatin sample for analysis at step 4 of this section.
   b. Sonicate the cell lysate with water bath sonicator. The following condition is optimized for HeLa cells when using the Qsonica Q800R.
   c. Transfer 50 µL to 0.7 mL of lysate into each sonication tube. Sonicate in a 4 ℃ water bath at setting with 65% power, 15 seconds ON, 45 seconds OFF pulse intervals for 2 hrs. (actual sonication ON time, total process time is 8 hours).

- *The efficiency of sonication depends upon cell type, cell equivalents and instrumentation. When possible, consult your instruments operators manual for guidelines. To provide a visual reference, an example of sonicated HeLa cell chromatin suitably fractionated for use with ChIRP kit is shown in Figure 7.*
- *Keep sonicator water bath ice cold. Sonication produces heat, which can denature the chromatin. Allow at least 45 seconds between sonication cycles to prevent sample overheating, which can damage RNA and DNA. Note that aliquots of lysate in different tubes often sonicate at different rates. Pool and redistribute aliquots of like samples into different tubes every 30 minutes to ensure homogeneity if possible.*
- *Cells cross-linked with glutaraldehyde take significantly longer to sonicate than cells treated with formaldehyde*
- *Polystyrene tubes tend to crack after prolonged sonication with the Qsonica Q800R sonicator. Replacing the tubes after each hour of sonication (or 4 hour total process time) is recommended*

2. Centrifuge the sonicated cell lysate at 16,100 x g at 4°C for 10 minutes.
3. Combine supernatants. Transfer 1 mL aliquots of the supernatant into new micro centrifuge tubes. Snap-freeze the tubes in liquid nitrogen and stored at -80° C. If desired, remove 5 µL of the lysate for microfluidic electrophoresis (e.g. Agilent Bioanalyzer) or agarose gel analysis of sheared DNA.
    - *Store on ice if the analysis will be done in the same day; otherwise store the sample at - 80ºC.*
    - *Sheared cross-linked chromatin can be stored at -80℃ for up to 3 months.*
    - *Avoid additional freeze-thaw cycles to prevent protein and RNA degradation.*
4. (Optional) Analyze the unsheared and sheared chromatin samples taken from step 1a and 3 of this section as described in steps V to XI of Appendix A.

E. Chromatin Isolation by RNA purification

Key Considerations Before Starting This Section

- **Reaction Size**

  For a typical ChIRP reaction 1 mL of sonicated cell lysate is diluted with 2 mL of Complete Hybridization Buffer in 15 mL conical tube. The amount of sonicated cell lysate and reaction size can be optimized for each experiment as long as volume ratio of sonicated cell lysate to Complete Hybridization buffer is kept at 1:2. For sample volumes less than 1.5 mL, microcentrifuge tubes can be used.

- **Amount of Probe**

  For a typical ChIRP reaction 100 pmol of total probe (2 µL of 50 µM total probe) per 1 mL sonicated cell lysate is recommended.
  Re-verify the concentration of probes that have been stored for a long time by Nanodrop (50 µM probes should spec ~250-300 ng/µL using single strand DNA setting).

- **Streptavidin Magnetic Beads**

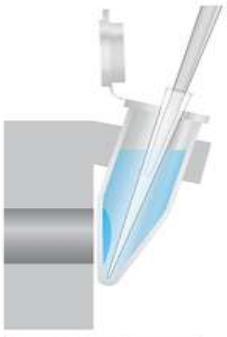
  The use of 120 µL of Streptavidin Magnetic beads per 100 pmol of probes is recommended. Additional beads may increase recovery of abundant target RNA complexes.

1. Label a sufficient number of 15 mL conical tubes for the desired number of ChIRP reactions.
2. Thaw tubes of sonicated cell lysate prepared in Section D at room temperature.

3. Remove 10 µL of sonicated cell lysate and place it into a new microcentrifuge tube labeled as "**RNA input**". Remove another 9 µL of sonicated cell lysate transfer it into a microcentrifuge tube labeled as "**DNA input**". Keep both input samples on ice until Section F, for RNA or Section G, for DNA isolation. These samples represent 10% RNA input and 1.0% DNA input, respectively.
4. Warm Hybridization Buffer (Part # CS216571) to 37 ℃ to disolve any precipitates. Pre pare 2 mL of Complete Hybridization Buffer for each mL of sonicated cell lysate. Add 300 µL of formamide to 1.7 mL of Hybridization Buffer (Part # CS216571), followed by 10 µL of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML) plus 10 µL of RNase inhibitor (Part # CS216144). Mix well.
5. Transfer 1 mL sonicated lysate to each 15 mL conical tube.
6. Add 2 mL Complete Hybridization Buffer to each tube and mix well.
7. Thaw probes at room temperature.
8. Add 2 µL of 50 µM probe (100 pmol of probe) to each tube and mix well.
9. Incubate at 37 ℃ for 4 hrs with end-to-end mixi ng or vertical rotation to hybridize probes.
10. Approximately 20 minutes before the end of the hybridization period, prepare Streptavidin Magnetic Beads.
    a. Label a sufficient nummber of 1.5 mL microcentrifuge tubes for the number of ChIRP reactions being performed (even, odd, and negative controls).
    b. Completely disperse and re-suspend Streptavidin Magnetic Beads (Part # CS220044) by pipetting or end-over-end rotation. No clumps of beads should be visible.
    c. Transfer 120 µL of Streptavidin Magnetic Beads to each of the labeled microcentrifuge tube prepared in "step a" above.
    d. Add 1 mL of Lysis Buffer (Part# CS216587) to each microcentrifuge tube and mix the beads by gently pipetting up and down several times to completely resuspend beads. Place the tube on the magnetic separator (e.g. Millipore Cat. # 20-400) for 1 minute.

Magna GrIP™ Rack
Cat. # 20-400

    e. Remove the supernatant making sure not to aspirate any magnetic beads. Remove the microcentrifuge tubes from the magnet.
    f. Repeat step d and Step e for two additional washes.
    g. Prepare a sufficient amount of Complete Lysis Buffer (100 µL per ChIRP) by supplementing 99 µL of Lysis Buffer (Part # CS216587) with 0.5 µL of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML) and 0.5 µL of RNase inhibitor (Part # CS216144).
    h. Resuspend the magnetic beads in each tube in 100 µL of Complete Lysis Buffer.


Supernatant can be removed gently and easily with no sample loss.

11. After 4 hr. hybridization reaction, add 100 µL of Streptavidin Magnetic Beads prepared in step 10 to each ChIRP reaction tube. Mix well.

12. Incubate at 37 ℃ for additional 30 minutes wit h constant mixing or rotation.
13. During the incubation pre-warm the Wash Buffer (Part # CS216569, 5 mL per reaction) to 37 ℃ and add 25 µL of 200X Protea se Inhibitor Cocktail III per 5 mL Wash Buffer.
14. Centrifuge the ChIRP reactions briefly to remove liquid from cap and sides of the 15 mL conical tube.
15. Place on a magnetic separator that will accommodate 15 mL conical tubes (e.g. cat. #LSKMAGS15, or #20-400) for 5 minutes.
16. Discard the supernatant, being careful not to disturb the magnetic beads.
17. Remove tubes from the magnet.

PureProteome™
Magnetic Stand
Cat. # LSKMAGS15

18. Wash the beads 4 times using 1 mL of prewarmed wash buffer for each wash.
    a. Add 1 mL pre-warmed Wash Buffer prepared in step 13 and mix the beads by gently pipetting several times to completely resuspend beads. Transfer the beads suspension to a new 1.5 mL microcentrifuge tube. (1st wash)
    b. Incubate at 37℃ with mixing for 5 minutes. Cent rifuge the microcentrifuge tubes briefly to remove liquid from cap and sides of microcentrifuge tubes.
        - *Mixing and 37°C incubation can be accomplished using an Eppendorf Thermomixer® system, a Labnet Shaking incubator, or a standard roller bottle hybridization oven.*
    c. Place tubes on a magnetic separator for 1 minute then carefully discard supernatant.
    d. Remove tubes from magnetic separation device. Add 1 mL pre-warmed Wash Buffer prepared at step 13 and mix the beads by gently pipetting several times to completely resuspend beads. (2nd wash)
    e. Incubate at 37℃ with mixing for 5 minutes. Cent rifuge the microcentrifuge tubes briefly to remove liquid from cap and sides of microcentrifuge tubes.
    f. Place tubes on a magnetic separation device for 1 minute then discard supernatant.
    g. Repeat steps d though f two additional times. (3rd and 4th wash)
        - *Be careful not to aspirate magnetic beads when discarding supernatant.*
19. Remove tubes from the magnet. Add 1 mL pre-warmed Wash Buffer prepared in step 13 and mix the beads by gently pipetting several times to completely resuspend beads. (5th wash)
20. Label a sufficient number of 1.5 mL microcentrifuge tubes for RNA isolation equal to the number of ChIRP reactions performed.
21. Transfer 100 µL of bead suspension from each reaction into the labeled 1.5 mL microcentrifuge tubes labeled above. This sample will be used for RNA isolation. The remaining 900 µL of bead suspension will be used for DNA isolation.
22. Incubate all tubes at 37 ℃ for 5 minutes with mixing.
23. Centrifuge all microcentrifuge tubes (both for RNA isolation and DNA isolation) briefly and place on a magnetic separation stand for 1 minute.
24. Discard supernatant.
25. Centrifuge all microcentrifuge tubes briefly a final time and completely remove remaining wash buffer with a sharp 10 -20 µL pipette tip.
26. Place tubes on ice and immediately proceed to RNA isolation and DNA isolation.

F. RNA Isolation

Important: Be sure to use the RNA-specific buffer indicated for this section. This section suggests the use of a QIAGEN miRNeasy® Mini Kit as part of the clean-up protocol. This kit is not supplied with this kit and can be obtained from QIAGEN Inc. While other RNA clean-up kits may be appropriate the miRNeasy kit has been demonstrated to work with this protocol.

1. Place the 100 µL bead samples (for RNA isolation) collected in **section E** step 26 and the 10 µL RNA input sample (10% Input) from **section E** step 3 on ice.
2. Resuspend each 100 µL bead sample with 95 µL Proteinase K Buffer for RNA (Part. # CS216567).
3. Resuspend the 10 µL RNA Input sample with 85 µL Proteinase K Buffer for RNA (Part #CS216567).
4. Add 5 µL Proteinase K (Cat. # CS207286) to each tube.
5. Incubate the microcentrifuge tubes at 50℃ for 4 5 minutes with end-to-end mixing.
6. Centrifuge the microcentrifuge tubes briefly and incubate for 10 minutes at 95℃.
7. Place the microcentrifuge tubes on ice for 2 minutes.
8. Centrifuge the microcentrifuge tubes briefly and add 0.5 mL of Trizol® Reagent to each tube. Pipette up and down several times to mix completely.
9. Incubate at room temperature for 10 minutes. Store at -80℃ or proceed to step 10.
10. Add 100 µL of chloroform to each tube.
11. Vortex vigorously for 15 seconds.
12. Centrifuge at 16,100 x g for 15 minutes at 4℃.
13. Remove the upper aqueous phase (~400 µL) and transfer to a new microcentrifuge tube.
    - *Transfer the same volume of upper phase for each of your samples.*
14. Add 600 µL (or 1.5 volume) of 100 % ethanol to each tube and mix well.
15. Pipette up to 700 µL sample, including any precipitate, into a QIAGEN miRNeasy® Mini column in a 2 mL collection tube. Close the lid and centrifuge at ≥8,000 x g for 15 s at room temperature. Discard the flow-through. Repeat above with the remainder of the sample.
16. Wash the columns once with 700 µL of Buffer RWT and twice with 500 µL of Buffer RPE.
    a. Add 700 µL Buffer RWT to the miRNeasy Mini column. Close the lid, and centrifuge for 15 seconds at ≥8,000 x *g*. Discard the flow-through.
       - *Add ethanol (96–100%) to Buffer RWT and Buffer RPE concentrates before use (see bottle label for volume).*
    b. Add 500 µL Buffer RPE to the miRNeasy Mini column. Close the lid, and centrifuge for 15 seconds at ≥8000 x *g*. Discard the flow-through.
    c. Add 500 µL Buffer RPE to the miRNeasy Mini column. Close the lid, and centrifuge for 2 min at ≥8,000 x g.
17. Place the miRNeasy Mini column into a new 2 mL collection tube. Centrifuge at full speed for 1 min to further dry the membrane.
18. Transfer the miRNeasy Mini column to a new 1.5 mL collection tube.

19. Pipette 30 µL nuclease-free water directly onto the miRNeasy Minicolumn. Close the lid, and centrifuge for 1 min at ≥8,000 x g to elude the RNA
20. Prepare 1 µL of 0.1M EDTA (Stop Solution) for each sample by diluting 0.5M EDTA (Pat #. CS203175) with 4 volumes of nuclease-free water.
21. Add 3 µL of 10X DNase I Reaction Buffer and 1 µL of DNase I (Pat # CS216565) to the eluted material from step 19 above.
22. Incubate all samples for 20 minutes at 37℃.
23. Centrifuge the tubes briefly and add 1 µL of Stop Solution prepared in step 20.
24. Incubate all samples for 10 minutes at 65℃.
25. Centrifuge the tubes briefly and place tubes on ice. Proceed to Section H, qRT-PCR analysis, to confirm RNA retrieval or use material to prepare RNA sequencing libraries (RNA-seq) if desired.

G. DNA Isolation

1. Place the 900 µL bead samples (for DNA isolation) from **section E** step 26 and the 9 µL DNA input sample (1% Input) from **section E** step 3 on ice.
2. Prepare a sufficient amount of Complete DNA Elution Buffer by adding 1.5 µL RNase A (Part # 20-297) and 1.5 µL RNase H (Part # CS216564) to 150 µL of DNA Elution Buffer (Part # CS216566) for each sample and DNA Input sample. Use table 3 to calculate the amount of buffer to prepare.

Table 3. Complete DNA Elution Buffer

Component	x 1	x reaction number
DNA Elution Buffer	150 µL	150 µL x_____=_____
RNase A	1.5 µL	1.5 µL x_____=_____
RNase H	1.5 µL	1.5 µL x_____=_____

3. Resuspend each 900 µL bead sample in 150 µL of Complete DNA Elution Buffer.
4. Resuspend the 9 µL DNA input sample in 141 µL of Complete DNA Elution Buffer.
5. Incubate all samples at 37 ℃ for 30 minutes wit h mixing.
6. Centrifuge the tubes briefly.
7. Place RNase treated 900 µL bead sample beads on a magnetic separation stand for 1 minute.
    - *Put the **DNA Input** sample aside. This will be used at step 11.*
8. Carefully transfer the supernatant to new 1.5 mL microcentrifuge tubes and place on ice. (1st elution)
9. To ensure complete DNA elution from beads, prepare a second aliquot of Complete DNA Elution Buffer with RNase A and RNaseH exactly as done in step 2 above.
10. Resuspend each sample of RNase treated beads in 150 µL of Complete DNA Elution Buffer.
11. Add 150 µL of Complete DNA Elution Buffer to **DNA input** sample from step 7.
12. Incubate all samples at 37 ℃ for 30 minutes wi th mixing.
13. Centrifuge the tubes briefly and place beads samples on the magnetic separator for 1 minute.
14. Carefully transfer the supernatant (2nd elution) to the tubes containing the 1st elution from step 8 above. (total volume of each sample should be 300 µL)

15. Add 15 µL Proteinase K (Cat. # CS207286) to each microcentrifuge tube of eluted sample and DNA input sample.
16. Incubate at 50 ℃ for 45 minutes with mixing.
17. Centrifuge the tubes briefly and place them at room temperature.
    - Immediately before use pre-spin Phase-Lock gel tubes (5 PRIME, Cat. # 2302810) at 12,000 - 16,000 x g for 20 to 30 seconds.
18. Transfer the samples to phase-lock gel tubes.
19. Add 300 µL Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 8.0) per sample.
20. Shake vigorously for 10 min.
21. Centrifuge at 16,100 x g for 5 minutes at 4 ℃.
22. Remove the upper aqueous phase (~300 µL) and place it in a new 1.5 mL microcentrifuge tube.
23. Add co-precipitant (2 µL Pellet Paint® NF, Cat.# 70748-3; or 5 µL of linear acrylamide) followed by 30 µL of 3M sodium acetate and 900 µL of 100% ethanol. Mix well.
24. Store the tubes at -20 ℃ overnight.
25. Centrifuge the tubes at 16,100 x g for 30 minutes at 4 ℃ and remove the supernatant being careful not to disturb the pellet.
26. Wash the pellet once by adding 1 mL of ice-cold 75% ethanol. Centrifuge at 16,100 x g for 5 minutes at 4℃. Carefully discard the supernatant a nd allow pellets to air dry.
27. Resuspend DNA pellet in 30 µL of 10 mM Tris-HCl, pH 8.5.
28. DNA samples are ready for analysis by qPCR (Step H) or for preparation of DNA sequencing libraries (DNA-seq).

H. Analysis of RNA retrieval and DNA binding by quantitative PCR

RNA and DNA isolated using the ChIRP kit can be analyzed by quantitative PCR. Once successful RNA retrieval and DNA binding (if the binding sites are known) to targets by ChIRP can be confirmed, further interrogation of DNA target in the complex may be pursued by population-based methods such as comparative microarray or deep sequencing.

Presented below are illustrative methods for performing real time quantitative measurement of ChIRP RNA retrieval and DNA binding using the control probes supplied in the EZ- Magna ChIRP kit (Cat. # 17-10495). Verification of retrieval of RNA and DNA binding can be performed using the relative standard curve method of qPCR analysis to compare RNA or DNA from negative control probes (LacZ) vs. positive control probe (TERC), or can alternatively be compared using the comparative Ct (ΔΔCt) method with two PCR amplicons, a positive control RNA (TERC gene) and a negative control binding RNA (GAPDH gene), or positive control DNA (WNT precursor region) and negative control DNA (GAPDH coding region). Input RNA or DNA is required whether using relative standard curve method or the comparative Ct (ΔΔCt) method. An example of successful retrieval of RNA is shown in Figure 2 (TERC RNA), and an example of DNA binding is shown in Figures 3 (WNT1 precursor region) by ChIRP with TERC RNA target. Another example of the ChIRP assay with lncRNA NEAT1 probes (Cat. # 03-308) are shown in Figure 4 (NEAT1 RNA) and Figure 5 (NEAT1 coding region). The NEAT1 assay was designed based on a publication by Simon MD, et al[3].

I. 1-Step Real-time Quantitative RT-PCR for RNA

1. Add 2 µL of the RNA sample to the PCR plate suitable for your real-time instrument of choice (Performing a triplicate of qPCR reactions per ChIRP sample is recommended).
   - *35 µL from all ChIRP or input sample are available.*
   - *2.0 µL or less ChIRP RNA is recommended for a 20 µL RT-qPCR reaction.*
   - *Performing triplicate of qPCR reactions per Nuclear RIP sample is also recommended.*
   - *If using the relative standard curve method, perform four 5- or 10-fold serial dilutions using the RNA from the 10% input sample, and use these samples to build a standard curve. Concentration of the ChIRP samples can be calculated as percent of input using the standard curve. Alternatively, data can be calculated in relation to cell equivalents or mass of purified RNA, if desired.*
2. Prepare a master reaction mix as shown in Table 4. Dispense enough reagents for one extra tube to account for loss of volume. For reagents other than iTaq™ Universal one-Step Kits (Bio-Rad) follow manufactures recommendations.
3. Add 18 µL of qPCR mix to 2 µL of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.

Table 4. 1-Step qRT-PCR setup and running parameters using iTaq Universal One-Step Kit

1-Step qRT-PCR reagent assembly for 1 reaction:

SYBR® Green Master Mix	10.0 µL
Reverse Transcriptase	0.25 µL
ddH_2O	6.75 µL
Primer mix	1.0 µL
Total	18 µL

qPCR parameters:

cDNA Synthesis	50℃ 10 min	
Polymerase Inactivation	95℃ 1 min	
Denature	95℃ 10 sec	40 cycles
Anneal and Extend:	60℃ 30 sec	

II. Real-time Quantitative PCR for DNA

1. Add 2 µL of the DNA sample to the PCR plate suitable for your real- time instrument of choice (Performing a triplicate of qPCR reactions per ChIRP sample is recommended).
   - *30 µL is available for analysis for each ChIRP reaction or Input.*
   - *2.5 µL or less ChIRP DNA is recommended for a 25 µL PCR reaction.*
   - *Performing triplicate of qPCR reactions per Nuclear RIP sample is also recommended.*
   - *If using the relative standard curve method, perform four 5- or 10-fold serial dilutions using the DNA from the 1 % input sample, and use these samples to build a standard curve. Concentration of the ChIRP samples can be calculated as percent of input using the standard curve. Alternatively, data can be calculated in relation to cell equivalents of mass of purified DNA, if desired.*
2. Prepare a master reaction mix as shown in Table 4. Dispense enough reagents for one extra tube to account for loss of volume.
3. Add 23 µL of qPCR mix to 2 µL of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.

Table 5. qPCR reagent setup and running parameters for DNA Analysis

qPCR reagent assembly for 1 reaction:		**qPCR parameters:**		
SYBR® Green Master Mix	12.5 µL	Initial Denature	95℃ 3 min	
ddH_2O	9.5 µL			
Primer mix	1.0 µL	Denature	95℃ 15 sec	50 cycles
Total	23 µL	Anneal and Extend:	60℃ 1 min	

I. RNA/DNA qPCR Data Analysis

There are many algorithms to analyze ChIRP result; the two most common methods are the relative standard curve method and the ΔΔCt method.

a. Normalize RNA concentration to percent of input using relative standard curve

1. For each RNA of interest, make four 5- or 10-fold serial dilutions with the 10% (RNA) or 1% (DNA) input sample, perform quantitative real-time PCR (or RT-PCR) with these input samples, ChIRP samples with negative control probes.
2. Calculate the threshold cycle (Ct) values using real-time detection system software from qPCR equipment manufacturer.
3. Use the threshold cycle (Ct) values of these input samples to build a standard curve.
4. Determine the concentration (C) of the ChIRP sample as percent of input using the standard curve.
5. Determine the fold enrichment by calculating the ratio of $C_{\text{probe of interest}}$ and $C_{\text{negative control probe}}$.
    - *For each independent experiment, we suggest that you perform the following ChIRP qPCR assays in triplicates in the same plate, if possible.*
    - *For the positive control experiment, the probe pool of interest is the TERC probe pools provided in the kit, the negative control RNA is human GAPDH (primers provided) and the positive control RNA is human TERC (primers provided), and the negative control DNA is human GAPDH coding region(primers provided) and the positive control DNA is human WNT region (primers provided),*

ChIRP Sample	Negative Control	Positive Control
Input dilution series 1	X	X
Input dilution series 2	X	X
Input dilution series 3	X	X
Input dilution series 4	X	X
ChIRP with probe pool (even) of interest	X	X
ChIRP with probe pool (odd) of interest	X	X
ChIRP with negative control probe pool (LacZ)	X	X

b. ΔΔCt method

1. Perform quantitative real-time PCR (or RT-PCR) with 2 µL of ChIRP Sample, and input sample in triplicates.
2. Perform quantitative real-time PCR (or RT-PCR) with primer set targeting a positive control and primer set targeting a negative control separately.
3. Calculate the threshold cycle (Ct) values using real-time detection 3.0 system software from qPCR equipment manufacturer.
4. Normalize ChIRP sample Ct values to input (ΔCt) for both ChIRP with probe of interest and with negative control probe by subtracting the Ct value obtained for the input from the Ct value for ChIRP sample: $\Delta Ct = Ct_{ChIRP} - (Ct_{input}\text{-Log2 [Input Dilution Factor]})$ (Input dilution factor is 10 if using 10% input sample).
5. Calculate the percent of input for each ChIRP sample: $\%Input = 2^{(-\Delta Ct\ [normalized\ ChIRP])}$.
6. Normalize ChIRP with probe of interest ΔCt values to negative control probe (ΔΔCt) by subtracting the ΔCt value obtained for the antibody of interest from the ΔCt value for negative control antibody ($\Delta\Delta Ct = \Delta Ct_{positive} - \Delta Ct_{negative}$).
7. Estimate the fold enrichment of the probe of interest in ChIRP sample over the negative control probe: Fold enrichment $= 2^{-\Delta\Delta Ct}$.

J. ChIRP-seq (NGS Analysis)

A ChIRP-seq NGS library can be generated using the recovered ChIRP DNA and an appropriate NGS library construction kit or method. If the ChIRP DNA sample is less than 1 ng, the PureGenome™ Low Input NGS Library Construction Kit (Cat. # 17-10492) or equivalent library construction kit can be used.

To ensure efficient library construction, ChIRP DNA can be column purified with MiniElute® Reaction Cleanup kit (QIAGEN) before library construction. The analysis of ChIRP-Seq library can be performed between a ChIRP DNA and input DNA (total DNA), between different probes, or between different stimulated condition of the cell lysates, etc. It may not be possible to perform analysis using the negative control (LacZ) in the kit because of very low amount of DNA typically recovered. An example of successful ChIRP-seq analysis with lncRNA probe sets NEAT1 (Cat. # 03-308) is shown in Figure 6.

Summary Protocol for Experienced Users

If this is your first time using this kit please follow detailed protocol above for best results. Once you are comfortable with all of the steps of the protocol this summarized version can be used.

I. Cross linking

1. **Prepare cells**. 2 x 10^7 cells. Stimulate or treat, if necessary.
2. **Trypsinize and harvest**.
3. **Wash the cells** with 20 mL of PBS
4. **Pellet the cells** by centrifuging at 800 x g for 5 minutes.
5. **Aspirate supernatant**.
6. **Resuspend cell pellet** in 20 mL of 1 % glutaraldehyde PBS per 2 x 10^7 cells. Invert the tube several times to mix.
7. **Incubate at room temperature for 10 minutes** on an end-to end shaker or rotator.
8. **Add 2 mL of 10X glycine** (Part # CS207370) to each 20 mL of 1 % glutaraldehyde PBS and invert the tube several times.
9. **Incubate at room temperature for 5 minutes** on an end-to end shaker or rotator.
10. **Pellet the cells** by centrifuging at 2000 x g for 5 minutes at 4º C.
11. **Aspirate supernatant**, removing as much medium as possible.
12. **Wash the cells** once with 20 mL of cold PBS.
13. **Pellet the Cells by** Centrifuging at 2000 x g for 5 minutes at 4º C.
14. **Aspirate supernatant carefully**.
15. **Resuspend the cell pellet with cold PBS.** (1.0 ml per **1 x 10^7** cells)
16. **Transfer each ml of cells in PBS** to a 1.5 mL micro centrifuge tube.
17. **Pellet Cells** by centrifuge at 2000 x g for 3 minutes at 4º C.
18. **Carefully remove supernatant** to avoid aspiration of cells with pipette tip
    - Cell pellet can be snap-frozen in liquid nitrogen and stored at -80°C, or continue on to cell lysis.

II. Cell Lysis

1. **Thaw cell pellets**.
2. **Pellet the cells** by centrifuging at 2000 x g for 3 minutes at 4 ℃.
3. **Remove any remaining PBS.** Use a sharp 10-20 µL pipette tip.
4. **Weigh each** pellet.
5. **Prepare Complete Lysis Buffer.** Add 5 µL of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML) and 5 µL of RNase inhibitor (Part # CS216144) to 1.0 mL of the Lysis Buffer (Part # CS216587) for each 100 mg cell pellet. Mix well.
6. **Resuspend each 100 mg cell pellet in 1.0 mL of Complete Lysis Buffer**.
7. Proceed immediately to Sonication to Shear DNA.

III. Sonication to Shear DNA

1. **Sonicate the cell lysate** with water bath sonicator.
2. **Centrifuge the sonicated cell lysate** at 16,100 x g at 4°C for 10 minutes.
3. **Combine supernatants**. Transfer 1 mL aliquots of the supernatant into new micro centrifuge tubes. Snap-freeze the tubes in liquid nitrogen and stored at -80°C.
4. **Analyze sheared chromatin by agarose electrophoresis. (Optional)**

IV. ChIRP

1. **Label 15 mL conical tubes** for the number of desired ChIRP reactions.
2. **Thaw sonicated cell lysate** at room temperature.
3. **Remove sonicated cell lysate,** 10 µL for **RNA input** and 9 µL for **DNA input**. Store both input samples on ice.
4. **Prepare Complete Hybridization Buffer**. Warm Hybridization Buffer (Part # CS216571) to 37 ℃ to disolve any precipitates. Add 300 µL of formamide to 1.7 mL of Hybridization Buffer (Part # CS216571) then add **10 µL** of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML), and **10 µL** of RNase inhibitor (Part # CS216144) per 1.0 mL of sonicated cell lysate and mix well.
5. **Transfer 1 mL sonicated lysate** to each 15 mL conical tube from step 1.
6. **Add 2 mL Complete Hybridization Buffer** to each tube and mix well.
7. **Thaw probes at room temperature**.
8. **Add 100 pmol of probe** (2 µL of 50 µM probe) to each tube and mix well.
9. **Incubate the tubes at 37 ℃ for 4 hrs.** with end to end mixing or vertical rotation.
10. **Prepare the Streptavidin Magnetic Beads** with 20 minutes remaining for hybridization,
    a. **Prepare 1.5 mL microcentrifuge tubes** for the number of ChIRP reactions.
    b. Completely disperse and re-suspend Streptavidin Magnetic Beads (Part # CS220044).
    c. **Transfer 120 µL of Streptavidin Magnetic Beads** to each microcentrifuge tube prepared.
    d. **Wash the beads 3 times** with 1 mL of Lysis Buffer (Part # CS216587).
    e. **Remove the supernatant**.
    f. **Remove the tubes** from the magnet.
    g. **Resuspend the magnetic beads** in each tube in 100 µL of Complete Lysis Buffer by supplementing Lysis Buffer (Part # CS216587) with 0.5 µL of 200X Protease Inhibitor Cocktail III (Part # 535140-1ML) and 0.5 µL of RNase inhibitor for each tube (Part # CS216144).
11. **Add 100 µL of Streptavidin Magnetic Beads suspension** from step 10 to each 15 mL tube after 4 hr. hybridization reaction is complete.
12. **Incubate the tubes at 37 ℃ for additional 30 minutes** with mixing.
13. **Pre-warm Wash Buffer** (CS216569, 5 mL per reaction) to 37 ℃ and add 25 µL of 200X Protease Inhibitor Cocktail III per 5 mL Wash Buffer.
14. **Centrifuge the 15 mL tubes briefly.**
15. **Place the 15 mL tubes on a magnetic separator** for 5 minute.
16. **Discard the supernatant**.
17. **Remove the tubes from the magnet**.
18. **Wash the beads 4 times with 1 mL of pre-warmed wash buffer** supplemented protease inhibitor.
    - Transfer the beads to new 1.5 mL microcentrifuge tubes at the 1st wash
    - Incubate at 37 ℃ for 5 minutes with mixing for each wash.
19. **Resuspend the beads with 1 mL of pre-warmed wash buffer.**
20. **Prepare and label** appropriate number of 1.5 mL microcentrifuge tubes.
21. **Remove 100 µL of beads suspension** and place it into new 1.5 mL microcentrifuge tubes for RNA isolation. Remaining 900 µL of beads suspensions are for DNA isolation.
22. **Incubate all tubes at 37 ℃ for 5 minutes** with mixing.
23. **Centrifuge all tubes** briefly and place on a magnetic separator for 1 minute.
24. **Discard supernatant**.
25. **Centrifuge all tubes briefly** again.
26. **Remove the last bit of wash buffer completely** with a sharp 10 µL pipette tip.
27. **Place the tubes on ice** and immediately proceed to RNA isolation and DNA isolation.

V. RNA Isolation

1. **Take tubes for RNA isolation samples and 10 µL RNA input sample** from above section.
2. **Resuspend each beads sample** in 95 µL Proteinase K Buffer for RNA (Part. # CS216567).
3. **Add 85 µL of Proteinase K Buffer for RNA (Part. # CS216567)** to 10 µL RNA INPUT sample.
4. **Add 5 µL of Proteinase K** (Cat. # CS207286) to each tube.
5. **Incubate all tubes at 50 ℃ for 45 minutes** with end-to-end mixing.
6. **Centrifuge the tubes briefly and incubate the tubes for 10 minutes at 95 ℃** .
7. **Place the tubes on ice** for 2 minutes.
8. **Centrifuge the tubes briefly**. **Add 0.5 mL of Trizol® Reagent** to each tube and mix completely.
9. **Incubate the tubes at room temperature for 10 min.** Store the tubes at -80 ℃ or proceed to step 10.
10. **Add 100 µL of chloroform** to each tube.
11. **Vortex the tubes vigorously for 15 second**
12. **Centrifuge the tubes at 16100 x g for 15 minutes at 4℃** .
13. **Remove the aqueous phase** (~400 µL) carefully and place it in a new 1.5 mL microcentrifuge tube.
14. **Add 600 µL of 100 % ethanol** to each tube and mix well.
15. **Spin sample through** miRNeasy® Mini columns
16. **Wash the columns once with 700 µL of Buffer RWT and twice with 500 µL of Buffer RPE**
17. **Place the columns into a new 2 ml collection tube**. Centrifuge at full speed for 1 min to further dry the membrane.
18. **Transfer the RNeasy Mini column to a new 1.5 ml collection tube**.
19. **Elute RNA with 30 µL nuclease-free water**.
20. **Prepare 1 µL of 0.1M EDTA (Stop Solution)** for each sample by diluting 0.5M EDTA (Pat # CS203175) with nuclease-free water.
21. **Add 3 µL of 10X DNase I Reaction Buffer and 1 µL of DNase I (Pat #** CS216565**)** to each tube from step 19 above.
22. **Incubate all tubes for 20 minutes at 37℃**
23. **Centrifuge the tubes briefly and add 1 µL of Stop solution** prepared at step 20.
24. **Incubate the tubes for 10 minutes at 65℃**
25. **Centrifuge the tubes briefly and place the tubes on ice**. Proceed to qRT-PCR analysis to confirm RNA retrieval or preparation of RNA sequencing (RNA-Seq) library.

VI. DNA Isolation

1. **Take tubes for DNA isolation and 9 µL DNA input sample** from **section IV**.
2. **Prepare Complete DNA Elution Buffer** by adding 1.5 µL RNase A (Part # 20-297) and 1.5 µL RNase H (Part # CS216564) to 150 µL of DNA Elution Buffer (Part # CS216566) for each sample and DNA Input sample.
3. **Resuspend each beads sample in 150 µL of Complete DNA Elution Buffer**.
4. **Add 141 µL of Complete DNA Elution Buffer to DNA input sample.**
5. **Incubate all tubes at 37 ℃ for 30 minutes** with mixing.
6. **Centrifuge the tubes briefly**
7. **Place the tubes for beads samples on the magnetic separator for 1 minute**.
8. **Transfer the supernatant** to new 1.5 mL microcentrifuge tubes and place them on ice. (1st elution)
9. **Prepare a second aliquot of Complete DNA Elution Buffer** with RNase A and RNaseH exactly as done in step 2 above.
10. **Resuspend each beads sample** in 150 µL of Complete DNA Elution Buffer.
11. **Add 150 µL of Complete DNA Elution Buffer** to **DNA input** sample.

12. **Incubate all tubes at 37 ℃ for 30 minutes** with mixing.
13. **Centrifuge the tubes briefly and place beads samples on the magnetic separator for 1 minute**.
14. **Transfer the supernatant** (2nd elution) to the tubes containing the 1st elution from step 8 above. (total volume of each sample should be 300 µL)
15. **Add 15 µL Proteinase K** (Cat. # CS207286) to each tube of eluted sample and DNA Input sample.
16. **Incubate the tubes at 50 ℃ for 45 minutes** with mixing.
17. **Centrifuge the tubes briefly** and put them at room temperature.
18. **Pre-spin down phase-lock gel tubes** (5PRIME).
19. **Transfer the samples to phase-lock gel tubes**.
20. **Add 300 µL Phenol:Chloroform:Isoamyl Alcohol** to each tube.
21. **Shake the tubes vigorously for 10 min.**
22. **Centrifuge the tubes at 16100 x g for 5 minutes at 4 ℃.**
23. **Remove aqueous phase** (~300 µL) and place it in a new 1.5 mL microcentrifuge tube.
24. **Add 2 µL of co-precipitant**, **30 µL of 3M sodium acetate, and 900 µL of 100% ethanol to each tube**. Mix well.
25. **Store the tubes at -20 ℃ overnight.**
26. **Centrifuge the tubes at 16100 x g for 30 minutes at 4 ℃** .
27. **Remove the supernatant** being careful not to disturb the pellet
28. **Wash the pellet once** with 1 mL of ice-cold 75% ethanol. Centrifuge at 16,100 x g for 5 minutes at 4℃. Carefully discard the supernatant and allow pe llets to air dry.
29. **Resuspend DNA pellet** in 30 µL of 10 mM Tris-HCl, pH 8.5. DNA samples are ready for analysis by qPCR (Step J) or preparation of high-throughput sequencing libraries.

Example of Magna ChIRP Data Using TERC Probe Set

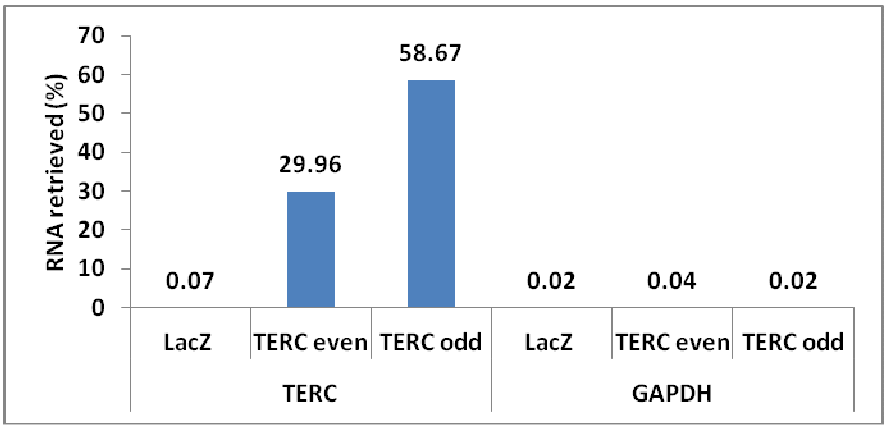


Figure 2: Successful retrieval of RNA by ChIRP with TERC probes

ChIRP was performed using HeLa cell lysate and either Magna ChIRP TERC lncRNA Probe Set even (Part # CS216575), odd (Part # CS216563) or Magna ChIRP Negative Control Probe Set (LacZ) (Part # CS216572). Purified RNA was then analyzed by qRT-PCR using RNA Positive Control Primers (TERC Gene, Positive target, Part # CS216598) and RNA Negative Control Primers (GAPDH) Part # CS216610).

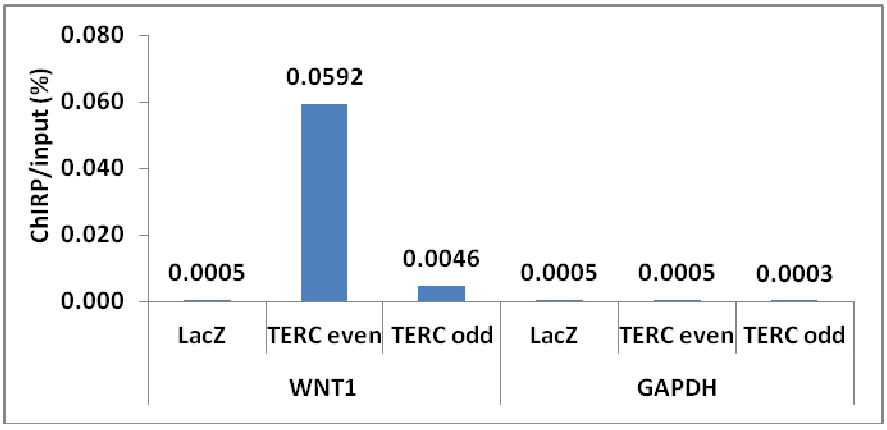


Figure 3. Successful DNA binding by ChIRP with TERC probes

ChIRP was performed using HeLa cell lysate and either Magna ChIRP™ TERC lncRNA Probe Set even (Part # CS216575), odd (Part # CS216563) or Magna ChIRP™ Negative Control Probe Set (LacZ, Part # CS216563). Purified DNA was then analyzed by qPCR using Magna ChIRP™ Primers, WNT-1 precursor (Positive target, Part # CS216609) and ChIRP Primers, GAPDH coding D2 (Negative Target, Part # CS207323).

Example of Magna ChIRP Data Using NEAT1 Probe Set

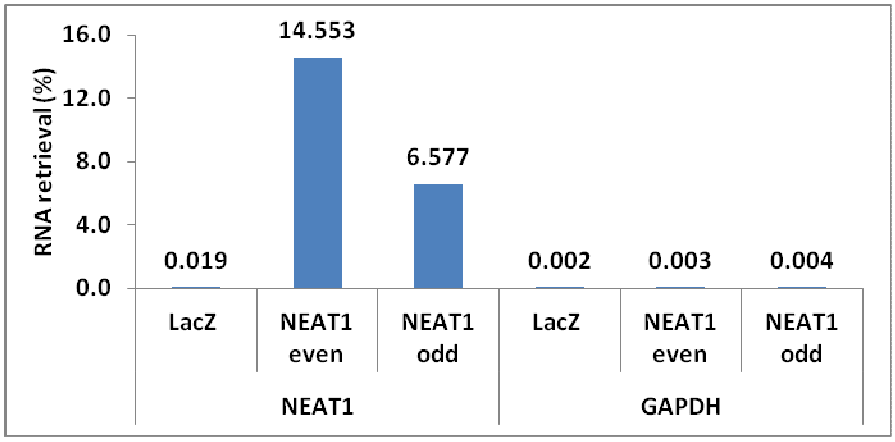


Figure 4: Successful retrieval of RNA by ChIRP with NEAT1 probes

ChIRP was performed using HeLa cell lysate and either Magna ChIRP NEAT1 lncRNA Probe Set even, odd (Cat. # 03-308) or Magna ChIRP Negative Control Probe Set (LacZ, Part # CS216572). Purified RNA was then analyzed by qRT-PCR using Primers specific for NEAT1 (Positive target) and GAPDH (Negative Target, Part # CS216610).

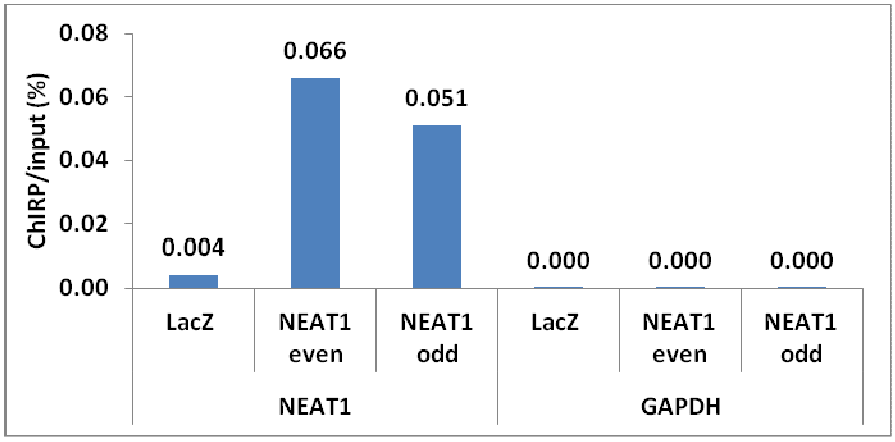


Figure 5. Successful DNA binding by ChIRP with NEAT1 probe sets

ChIRP was performed using HeLa cell lysate and either Magna ChIRP NEAT1 lncRNA Probe Set even, odd (Cat. # 03-308), or Magna ChIRP negative control probe set (LacZ, Part # CS216572). Purified DNA was then analyzed by qPCR using Primers specific for NEAT1 coding region and GAPDH coding D2 (Negative Target, Part # CS207323).

Example of Magna ChIRP-seq Data

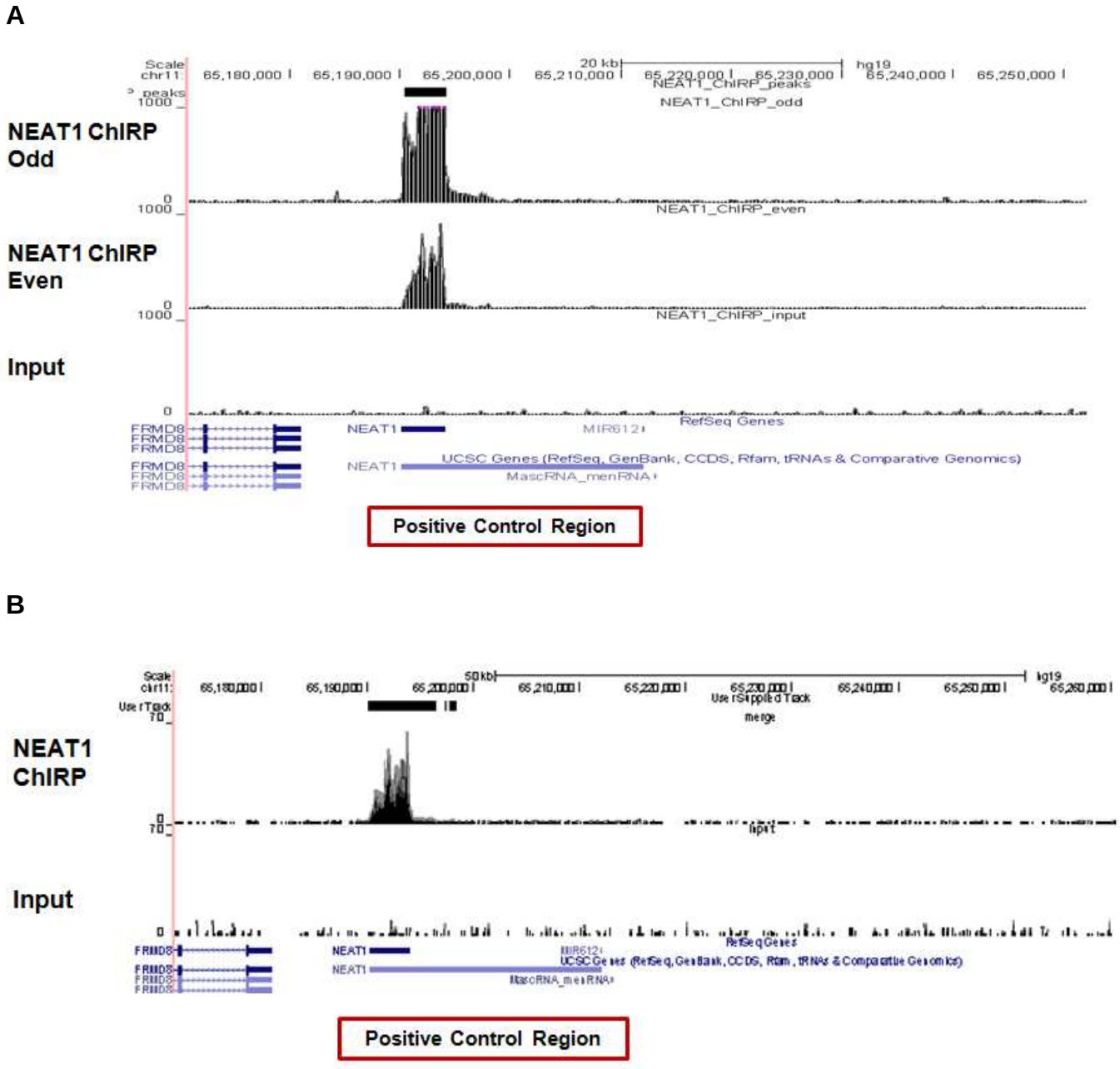


Figure 6: Successful DNA binding by ChIRP-seq with lncRNA probe sets NEAT1
ChIRP-seq was performed with lncRNA probe sets NEAT1 (Cat. # 03-308). The sequence libraries were constructed with NGS Library Construction Kit (Cat. # 17-10492) and sequenced on HiSeq instrument (Illumina). The sequence reads were aligned to the reference genome (hg19) using Bowtie. (A) Peaks were called separately using data from even and odd probe sets. Algorithms such as MACS can be used for this purpose. Those in common were considered to be valid peaks. (B) A series of post-alignment processing and filtering steps were carried out using analysis software available from the laboratory of Howard Chang (http://changlab.stanford.edu/protocols.html). The data showing localization of NEAT1 mRNA to the NEAT1 gene region.[3]

Appendix A: Optimization of Sonication

Optimal conditions for shearing cross-linked DNA to 100-500 base pairs in length depend on the cell type, cell concentration, and the specific sonicator and equipment settings, including the power settings, duration and number of pulses. Glutaraldehyde cross-linked cells take significantly longer to sonicate than those cross-linked with formaldehyde.

Approaches for optimizing sonication may include the following:

A. Choosing a fixed concentration of cell equivalents per mL of Lysis Buffer and varying cycles and/or power settings of sonication.
B. Varying the concentration of cell equivalents per mL of Lysis Buffer with constant sonication parameters.
C. A combination of both approaches. However changing more than one parameter at a time is not recommended.

The protocol presented below describes optimization by option A with a water bath sonicator (Q800R Sonicator, Qsonica) as a specific example.

I. Generate a Cross-linked cell pellets by following Section B.
II. Continue following the Cell Lysis procedure (section C) through Step 6 with 100 mg of cell pellet (approximately 10^7 cells in 1.0 mL of cell lysate).
III. Remove 5 µL cell lysate prior to sonication for analysis of unsheared DNA.
IV. Transfer <0.7 mL lysate in each sonication tube. Shear the cell lysate in a 4 ℃ water bath at setting with 65% power, with 15 seconds ON, 45 seconds OFF pulse intervals.
V. Remove 5 µL sonicated chromatin from each condition to a fresh micro centrifuge tube every 30 minutes.
    - *Sonicate the cell lysate until it is no longer turbid. This may take as little as 30 min and as much as 3 hr. sonication time (2 to 12 hours of total process time).*
    - *The efficiency of sonication depends on cell type, cell equivalents and instrumentation. When possible, consult your instrument manufacturer or operation manual for guidelines. To provide a visual reference, an example of sonicated HeLa cell lysate suitably fractionated for use with ChIRP kit is shown in Figure 7.*
    - *Keep sonicator water bath ice cold. Sonication produces heat, which can denature the chromatin. Allow at least 45 seconds OFF time per sonication cycle to prevent sample overheating which can damage RNA and DNA. Lysate aliquots in different tubes will likely sonicate at different rates, so pool and redistribute aliquots of the same sample in different tubes to ensure homogeneity if possible.*
VI. To each 5 µL sample (unsheared and sheared), add 90 µL Proteinase K Buffer for DNA, (Part # CS216568) and 5 µL of Proteinase K (part # CS207286).
VII. Vortex to mix and spin down briefly. Incubate the samples at 50℃ for 45 minutes.
VIII. Cool the samples down to room temperature.
IX. Centrifuge the tubes briefly.
X. Purified DNA with PCR Purification Kit (QIAGEN). Elute DNA in 30 µL Elution Buffer (EB, QIAGEN).
XI. Load and analyze 10 µL on a 1-2% agarose gel.

XII. Observe which of the shearing conditions gives a smear of DNA in the range of 100 -500 bp. See Figure 7 as an example.

XIII. Repeat optimization of the shearing conditions if the resulting DNA fragments are not in the desired size range. Increase the process time and/or power of the sonication if the chromatin DNA fragments are too large.

DNA Sonication: Sheared DNA Should Be Between 100-500 bp in Length

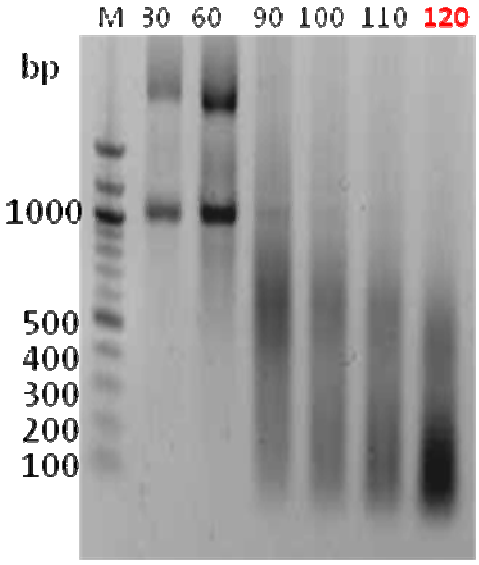


Figure 7: Chromatin Preparation

HeLa cells were cross-linked with 1.0% glutaraldehyde for 10 minutes.

Cells were lysed in Lysis Buffer. Time course experiments were performed with Q800R Sonicator (Qsonica). DNA was extracted by column after proteinase K digestion and analyzed by agarose gel. Cell lysate sonicated for 120 minutes (Lane 7) resulted in the best shearing for ChIRP experiments.

ChIRP Optimization and Troubleshooting

Step	Potential Problems	Experimental Suggestions
Cross-linking	Not enough or too much cross-linking	• Always use fresh unopened glutaraldehyde. • The amount of glutaraldehyde and time of cross-linking may be determined empirically. Conduct a time course at a fixed glutaraldehyde concentration and/or investigate a range of glutaraldehyde concentrations for a fixed time.
Cell Lysis	Inefficient disruption of cells	It is important to have sufficient Lysis Buffer for the weight of cells processed. Follow the guidelines in this protocol.
Chromatin Shearing	Not enough/too much sonication	If fragments are too large or too small, optimize sonication conditions using approach outline in appendix A to obtain appropriate size fragments.
	Denaturation of proteins from overheating sample	Keep the water bath cold during sonication. Shorten the duration of each sonication pulse and increase the number of sonication pulses. Allow sufficient time for sample to cool between pulses.
ChIRP	Probe doesn't retrieve RNA in the lysate	• Confirm the probe design • Check the expression of the target RNA. Target RNA should show Ct value of lower than 23 per 100 ng of total RNA by qRT-PCR. • Use Millipore ChIRP control probes • Perform ChIRP with different amount of probes for a fixed amount of lysate or vice versa. • Consider using more or less lysate (chromatin)
	Insufficient quantity of Streptavidin Magnetic Beads.	• The magnetic beads settle to the bottom of the tube over time. Make sure the magnetic beads are well mixed prior to beads withdrawal. • Carefully aspirate beads when using vacuum aspirator and use a high strength neodymium magnetic rack such as the Millipore Cat. # 20-400 Magna GrIP Rack. • Use more beads.
Washing	High background due to insufficient washing	• Ensure buffer is pre-warmed ChIRP washes is are at 37 ℃ • Increase number of washes.
	Low signal due to aspiration of the beads	• Carefully remove supernatant and make sure there are no beads in the supernatant prior to removing it. • Use rack with magnets capable of firmly holding beads in place (e.g. Magna GrIP Rack Cat. #. 20-400)
Elution and Proteinase K digestion	Incomplete elution	When performing elution, make sure that the temperatures are correct. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.

Step	Potential Problems	Experimental Suggestions
RNA Purification	Protein Contamination	• Avoid the interphase when extracting RNA using Trizol® extractions.
	Low RNA yield	• Most ChIRP reactions do not yield measureable amounts of RNA. Sub nanogram quantities of RNAs can however be detected by qRT-PCR. • If RNAs are not detectable following cDNA synthesis, consult ChIRP step troubleshooting above.
	RNA degraded	• Use RNase inhibitor in solutions as recommended in this protocol. Make certain that all work conditions are RNase-free and RNases are not being introduced. • Follow the guidelines in the RNase control section before the Detailed Protocol section. • Use RNase-inactivating reagents to ensure work area and materials are RNase-free.
	No RNA detected	• Confirm the probe designs are correct. • Confirm the expression of the RNA in the cell by qRT-PCR.
PCR	No PCR product from Positive Control ChIRP samples	• Increase amount of ChIRP sample used for PCR reaction up to 10% of total reaction volume. • Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR reaction with melting curve assessment to confirm amplification conditions and ability of primers to generate a single DNA product. • Confirm the probe designs are correct.
	High background level with negative control ChIRP samples	Insufficient wash after ChIRP. Increase the time of beads washing. More stringent washing may be achieved by adding optimally determined concentration of formamide or by increasing temperature.

Related Products

Product	Description	Catalog Number
Magna ChIRP™ Negative Control Probe Set	Negative control probe set for ChIRP	03-307
Magna ChIRP™ NEAT lncRNA Probe Set	Pre-designed even and odd probe set for ChIRP	03-308
Magna ChIRP™ TERC lncRNA Probe Set	Pre-designed even and odd probe set for ChIRP	03-309
Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays	17-10520
EZ-Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10521
Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays	17-10522
EZ-Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10523
Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10460
Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays	17-700
EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays, plus positive control antibody and control primers	17-701
EZ-Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions plus positive and negative control antibodies and validated qPCR primer set. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10461
RIPAb+™ Validated Antibody Primer Set	Proven for RIP or ChIP and lot tested for performance. See the complete selection at www.millipore.com/antibodies	Multiple
ChIPAb+™ Validated Antibody Primer Set		Multiple

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1. Chu C, Qu K, Zhong FL, Artandi SE, Chang HY. *Mol Cell*. 2011 Nov 18; 44(4):667-78.
2. Chu C, Quinn J, Chang HY. *J Vis Exp*. 2012 Mar 25;(61):e3912
3. Simon MD, Wang CI, Kharchenko PV, West JA, Chapman BA, Alekseyenko AA, Borowsky ML, Kuroda MI, Kingston RE. *Proc Natl Acad Sci U S A*. 2011 Dec 20;108(51):20497-502

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