Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit

Nuclear RIP (Cross-Linked) Kit (Catalog No. 17-10520)
EZ-Nuclear RIP (Cross-Linked) Kit (Catalog No. 17-10521)

24 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. Other immunoprecipitation methods, such as RNA-binding protein immunoprecipitation (RIP), have been developed to interrogate RNA binding proteins (either cytoplasmic or nuclear) associated with specific RNA molecules. Given that chromatin is composed of biomolecules that may interact with DNA binding proteins, DNA, as well as RNA binding proteins and RNA, methods such as nuclear RIP have emerged to allow isolation and identification of RNA molecules associated with chromatin. Nuclear RIP is typically done by interrogating chromatin prepared from cells or tissues of interest with an antibody against a chromatin-associated protein. The preparation of chromatin used for these experiments can be tailored to suit experimental requirements. The chromatin of interest can be prepared either in a native configuration (Nuclear RIP (Native))[1] or by using chemical cross-linking agents (Nuclear RIP (Cross-link))[2], and can further be fragmented using mechanical or enzymatic methods in the presence of RNAse inhibitors and utilizing DNAse I digestion. To enable researchers to easily use either approach, we offer Magna Nuclear RIP kits for both the native and cross-linked methods (see page 5 for ordering information).

Native nuclear RIP uses chromatin prepared from cells that have not been treated with any cross-linking reagents. The native procedure follows a simpler protocol with milder washing conditions that are designed to protect RNA-protein interactions. Wash conditions can be adjusted easily, and the potential for background is typically lower compared to cross-linking conditions. The milder wash conditions used with this method can increase the probability of detecting weak RNA-protein interactions.[3, 4] In addition, this method has been used to generate RIP-Seq data in multiple publications.[1, 3] Cross-linked nuclear RIP uses chromatin prepared from cells treated with a cross-linking agent (formaldehyde) to preserve protein:DNA, Protein:protein, and protein:RNA interactions. The cross-linked approach requires additional protocol steps and uses more stringent washing conditions and is the method of choice for detecting indirect interactions. Wash conditions cannot be easily adjusted, but their stringency ensures removal of background signal. This method is similar to a traditional chromatin immunoprecipitation (ChIP), and can be used to directly compare DNA-protein interactions and RNA-protein interactions using aliquots of the same starting material.

It is very likely that multiple proteins may be interacting with the RNA and chromatin, and the strength of these various chromatin RNA interactions may not be well characterized. In many cases the

analysis of targets using both native and cross-linked approaches may help more fully characterize these interactions and cross verify results.

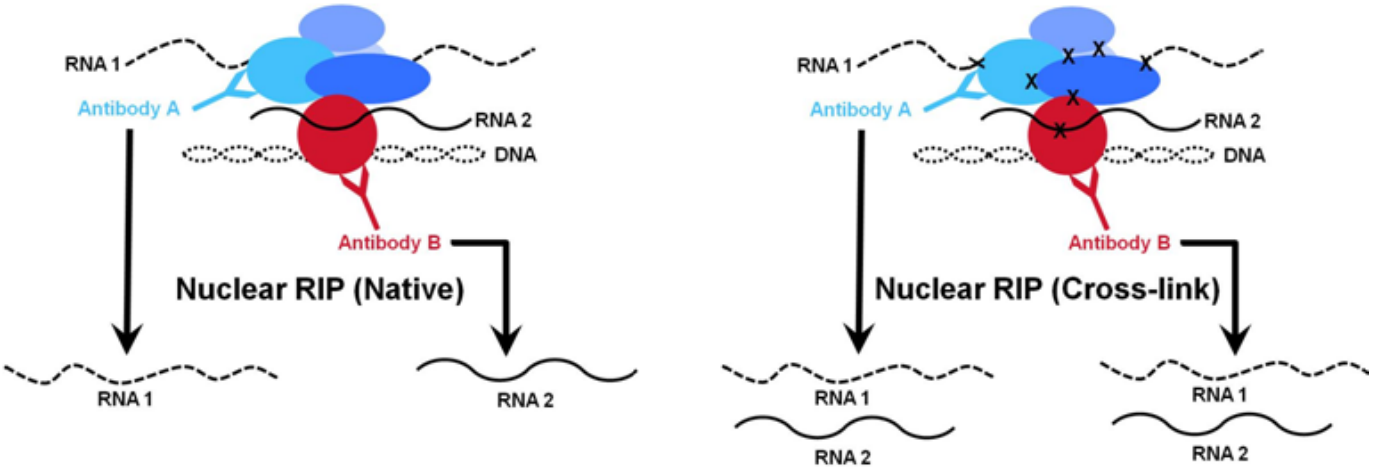

Figure 1. Mapping Protein:RNA interactions using the Magna Nuclear RIP (Native) vs. Magna Nuclear RIP (Cross-Linked) approach. Chromatin is prepared as the substrate for immunoprecipitation in both methods, although the details of the isolation and fragmentation procedure differ. Native RIP is expected to recover high affinity, more direct interactions between proteins encoded RNA binding motifs and candidate RNAs, whereas cross-linking can capture higher molecular weight complexes in *in vivo* configurations with possibly lower affinities.

In the Magna Nuclear RIP (both native and cross-linked) kits, we have designed reagents and developed optimized protocols to enable the study of RNA-protein interactions in the nuclei of cells. With this kit one can easily conduct experiments to discover and analyze non-coding RNA function, as well as profile mRNA molecules that may be associated with protein complexes in the nucleus. The reagents provided with these kits and methods described in the user manuals have been demonstrated to show improved signal-to-noise ratios, work with varied amounts of starting materials and enable downstream analysis by either qRT-PCR or Next-Generation Sequencing.

Comparison Magna Nuclear RIP (Native) versus Magna Nuclear RIP (Cross-Linked)		
Characteristic	**Native**	**Cross-link**
Analysis of high affinity Protein: RNA interactions	Yes	Yes
Analysis of low affinity Protein:RNA interactions	Possible by controlling wash stringency	Preferred method
Control of Wash Stringency to evaluate affinity of interactions	Preferred method	Not recommended
Ability to capture indirect RNA interactions in multi protein complexes	Possible by controlling wash stringency	Preferred method
Shearing method	Hydrostatic shearing/freeze thaw	Sonication

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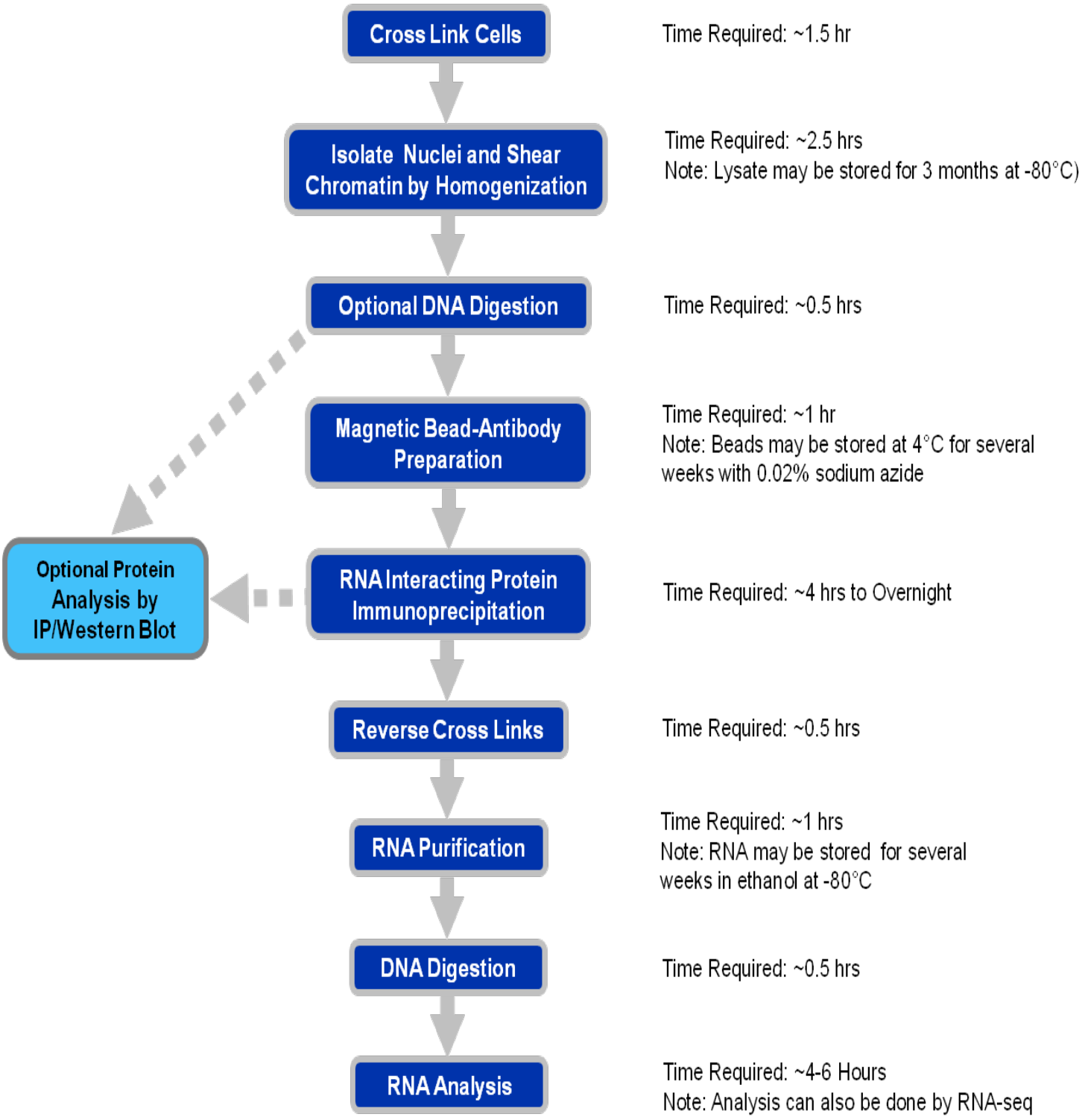
Magna Nuclear RIP (Cross-Linked) Kit Overview



Warnings and Precautions

***This protocol utilizes organic extraction, in which phenol is used. Contact with phenol causes burns and can be fatal. Use gloves and other personal protective equipment when working with phenol.

**The Protease Inhibitor Cocktail 200X contains dimethyl sulfoxide (DMSO) which can penetrate skin and mucous membranes upon contact. Use gloves and other personal protective equipment when working with the protease inhibitor cocktail.

Storage and Stability

Upon receipt, store components at the temperatures indicated on the labels.
Kit components are stable for 6 months from date of shipment when stored as directed.

Related Products

Product	Description	Catalog Number
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

Materials Provided (Kit Configurations)

The Magna Nuclear RIP (Cross-Linked) kit provides sufficient reagents for 12 individual chromatin preparations and 24 Nuclear RNA immunoprecipitations. The EZ-Magna Nuclear RIP (Cross-Linked) kit includes these reagents plus positive and negative control antibodies and a positive and a negative primer sets for qPCR analysis. Please refer to the table below for details on kit components.

MAGNARIP04 (Component box of all MAGNA Nuclear RIP Cross-Linked Kits) Store at 4 ° C		
Component	**Part #**	**Quantity**
10X Glycine	CS207370	27 mL
10X PBS	CS207371	26 mL
Nuclei Isolation Buffer	CS207355	6 mL
RIP Cross-Linked Lysis Buffer	CS216129	6 mL
Magna ChIP Protein A/G Magnetic Beads*	CS207374	250 µL
Nuclear RIP Dilution Buffer	CS216130	50 mL
Low Salt Wash Buffer	CS200625	12.5 mL
High Salt Wash Buffer	CS200626	12.5 mL
LiCl Wash Buffer	CS200627	12.5 mL
TE Buffer	CS200628	12.5 mL
RIP Elution Buffer	CS216131	10 mL
10% SDS	CS216132	1.0 mL
0.5 M EDTA	CS203175	500 µL

MAGNARIP05 (Component box of all MAGNA Nuclear RIP Cross-Linked Kits) Store at -20°C		
DNase I (RNase Free)	CS216133	200 µL
DNase I Supplement	CS216134	120 µL
DNase I Reaction Buffer	CS216135	75 µL
Protease Inhibitor Cocktail III, Animal Free **Contains DMSO	535140-1ML	1 mL
RNAse Inhibitor	CS216138	90 µL
Proteinase K (10 mg/mL)	CS207286	200 µL

MAGNARIP06 (Component box of 17-10521 only) Store at -20°C)		
Normal Mouse IgG	CS200621	125 µg
Positive Control Antibody (Anti-EZH2 Clone AC22)	CS203195	50 µL (1.0 µg/ µL)
NEAT1 Positive Control Primers FOR: 5'-CTT CCT CCC TTT AAC TTA TCC ATT CAC-3' REV: 5'-CTC TTC CTC CAC CAT TAC CAA CAA TAC-3'	CS216139	75 µL
U1 snRNA** Negative Control Primers FOR: 5'-GGG AGA TAC CAT GAT CAC GAA GGT-3' REV: 5'-CCA CAA ATT ATG CAG TCG AGT TTC CC-3'	CS203215	75 µL

**The magnetic beads described here are the same as those used in Magna ChIP A/G kits (see page 5). Magna ChIP A/G beads are validated for both ChIP and RIP protocols.*

Materials Required But Not Supplied

Reagents

- Cells, stimulated or treated as needed for the experimental system
- Antibody of interest for RNA-binding protein immunoprecipitation (RIP)
- Negative Control Antibody
- PBS (RNase free)
  (e.g. Fisher, Cat. # BP2438-4)
- TRIzol® LS Reagent (Life Technologies Cat. #10296-010)
- Chloroform (e.g. Fisher, Cat. # BP1145)
- 100% Ethanol (molecular biology grade)
- Isopropanol (molecular biology grade)
- Precipitation Carrier
  - Pellet Paint® Co-Precipitant (125reactions, EMD Millipore Cat. # 69049) or RNase-free glycogen
  - If sample are intended for Next Gen Sequence library preparation, use Linear Acrylamide (5mg/mL, Life Technologies Cat. # AM9520)
- Nuclease Free Water
- 10 mg/mL RNaseA
- 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 RNA-Seq Library Construction (optional)

- RNA-Seq library construction system (e.g. NuGEN Inc. Encore® Complete RNA-Seq DR Multiplex System 1-8 System Cat. # 0333-32, or a combination of Ovation® RNA-Seq System V2 Cat. # 7102-08 and Encore Rapid DR Multiplex System 1-8 Cat. # 0319-32)
- RNeasy® mini Kit (QIAGEN, Cat. # 74104)

Equipment

- Dounce homogenizer (loose pestle, necessary for tissue samples but optional for cultures cells)
- Sonicator
- Magnetic Separator
  Magna GrIP™ Rack (8 well, Millipore Cat. # 20-400) or PureProteome™ Magnetic Stand, (Millipore Cat. # LSKMAGS08)
- Vacuum Aspirator
- Vortex mixer
- Rotating wheel/platform
- Centrifuge for cell culture
- Microcentrifuge
- Ultra low temperature freezer
  (below -80°C)
- Thermomixer (60°C 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
- Centrifuge tube (15 mL and 50 mL)
- Nuclease-free Microcentrifuge tubes, 1.5 mL
- Real-time PCR thermal cycler
- PCR tubes, 0.2 mL

Detailed Protocol

Please read through the entire protocol and carefully plan your work before starting. This protocol is optimized for Nuclear RIP with chemically cross-linked, sonicated chromatin. If uncross-linked (native) chromatin is preferred, use the Magna™ Nuclear RIP (Native) kit (Cat. # 17-10522) or EZ- Magna™ Nuclear RIP (Native) kit (Cat. # 17-10523). The nuclear RIP 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 figure on page 4.

A. Planning Lysate Requirements for Cross-Linked Nuclear RIP Experiments

- Calculate the number of desired immunoprecipitations. Samples include the antibodies of interest and a negative control IgG of the same species as the antibody of interest (user supplied). Anti-Ezh2 (Part # CS203195) and negative control Normal mouse IgG (Part # CS200621) can be used as controls for the nuclear RIP procedure, Both components are included in the EZ-nuclear RIP (Cross-Linked) kit (Cat. # 17-10521).
- Typically one nuclear RIP (Cross-Linked) reaction (i.e. one immunoprecipitation using one antibody) requires 50 µL of Chromatin from ~1.0 x 10^6 cells**.** The calculation for the volume of Nuclei Isolation buffer and RIP Cross-Linked Lysis Buffer required for a RIP experiment is based upon the volume of the **cell pellet** harvested. This volume may vary based on the type of cells utilized. An example of the size of HeLa cell culture and required Nuclei Isolation Buffer and RIP Lysis Buffer are shown below (Table 1). In many cases the number of cells/volume or RIP lysate required can be adjusted. However, the recommended amount of chromatin should be used for initial experiments. Once you've demonstrated successful RIP with a candidate antibody in a certain cellular context, the amount of lysate per nuclear RIP reaction may be reduced or further optimized as necessary.
- The total number of the cells or total amount of protein used per RIP must be optimized based upon the abundance of the RNA-binding protein being investigated as well as the planned method of RNA detection.

Table 1. Approximate volumes of RIP lysis buffer per cell culture vessel (HeLa cells)

Type of vessel	Surface Area (cm^2)	Cell Number	Volume of Nuclei Isolation Buffer (µL)	Volume of RIP Cross-linked Lysis Buffer (µL)
T-75	75	~0.5 x 10^7	250	250
T-225	225	~1.3 x 10^7	650	650
10 cm plate	78.5	~0.5 x 10^7	250	250
15 cm plate	176.6	~1.0 x 10^7	500	500

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 to minimize introduction of RNAses. All instruments, glassware and plastic-ware 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 # CS216137) 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.

B. In Vivo Cross linking of Proteins to RNA/DNA

I. Cultured cells (Adherent or Suspension)

1. Prepare cells for chromatin isolation. Stimulate or treat, if necessary, adherent mammalian cells at ~80 to 90% confluence in a 150 mm culture dish containing 20 mL of growth media. Include one extra plate of cells to be used solely for estimation of cell number. If using suspension cells stimulate or treat if necessary in 20 mL volume.
    - *For HeLa cells, this is approximately 1 x 10^7 cells. This typically generates a preparation of chromatin sufficient for 10 separate immunoprecipitations using 1 X 10^6 cell equivalents/reaction.*
    - *The volume of buffers supplied in the kit is sufficient to generate chromatin from up to 12 x 15 cm plates of cultured cells.*
    - *Cell numbers can be scaled according to the performance of the antibody of interest to optimize highest signal-to-noise ratio relative to negative control (mock IgG or negative RNA control). For example, Anti- EZH2 (Part # CS203195) can perform successful Nuclear RIP (Cross-linked) reaction of as few as 5 X 10^3 HeLa cells. This protocol is written for simplicity using 1 X 10^6 cells per Nuclear RIP (Cross-linked) reaction to ensure optimal performance of the control antibodies.*
    - *Chromatin from other types of culture vessels can be isolated with slight modifications to the protocol.*
2. Prepare 22 mL of 1X PBS (2.2 mL 10X PBS and 19.8 mL water) for each 150 mm culture dish. Store on ice for washes in later steps of the protocol. It is important that this 1X PBS is ice cold.
3. Thaw the 200X Protease Inhibitor Cocktail III at room temperature for later use.
4. Add 165 µL of 37% formaldehyde (or 330 µL of 18.5% formaldehyde) directly to 20 mL of growth media to cross link. Gently swirl dish to mix.
    - *Final concentration of formaldehyde is **0.3%.** Use high quality (molecular-biology grade) formaldehyde. Formaldehyde is typically stabilized with methanol. Upon evaporation of methanol, a white precipitate is observed. Do not use formaldehyde if white precipitate is visible in the solution.*
    - *If using suspension cells, formaldehyde can be added to the media for a final concentration of **0.3%**. However, depending upon the volume of cell culture, there might not be sufficient 10X glycine to perform 12 chromatin preps. To ensure sufficient glycine for all chromatin preparations gently pellet the cells by centrifugation at1,350 X g for 5 min. and resuspend the cell pellet in 20 mL PBS or media for every 1 x 10^7 cells before adding formaldehyde.*
5. Incubate at room temperature (18-25ºC) for 10 minutes.
    - *Agitation of cells is not necessary. Performing cross linking in low serum conditions with culture media or PBS is optional, as an optimization parameter to improve cross linking efficiency. Cross linking time can be increased but may result in higher non-specific association of RNAs with the RIP antibody of interest.*
6. During the 10 minute incubation, prepare PBS/protease inhibitor mix to be used for adherent cell collection: Add 1 mL of ice cold 1X PBS to a conical tube for every dish and add 5 µL of the 200X Protease Inhibitor Cocktail III. Store on ice.
    - *Please note that Protease Inhibitor Cocktail III contains DMSO and will remain frozen below 18.4°C.*
    - *This step is only required for adherent cells that must be scraped from the plate. This step and preparation of this buffer is not required for suspension cells.*
7. Add 2 mL of 10X glycine to each dish to quench excess formaldehyde.
    - *If using suspension cells, make sure to add sufficient 10X glycine for a 1X final concentration.*
8. Swirl for 5 minutes at room temperature.
9. Place dishes on ice.

10. Aspirate media, removing as much medium as possible, being careful not to disturb the cells.
    - *If using suspension cells, spin down cells at 1,350 X g for 5 min. then carefully aspirate media.*
11. Add 10 mL of cold 1X PBS prepared in step 2 to wash cells.
12. Carefully remove 1X PBS and repeat wash.
    - *For suspension cells, spin down cells 5 min. at 1350 X g, carefully aspirate media and repeat wash.*
    - *Be sure to rinse the cells twice. If using suspension cells after second rinse, cell pellets can be snap-frozen or you can continue on to cell lysis and sonication (section C of this protocol).*
13. Add 1 mL of PBS/protease inhibitor mix prepared in Step 6.
14. Scrape cells from each dish into a separate 1.5 mL microcentrifuge tube.
15. Spin at 800 x g at 4°C for 5 minutes to pellet cells.
16. Carefully remove supernatant to avoid aspiration of cells. 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.

II. Fresh tissue

1. Dissect non-fixed fresh tissue. Transfer tissue sample into a 50 mL conical tube and wash twice with 30 mL ice cold 1X PBS. Weigh tissue and record weight (you will use this value to determine volume of buffer to use in section C of this protocol).
    - *A piece of tissue approximately 5 mm^3 contains around 10^7 cells and should be sufficient for 10 ChIP samples. Although the mass will depend on the cellularity of the tissue, 5 mm^3 of tissue is approximately 100-200 mg by mass.*
    - *Carefully handle and promptly process all tissue samples to preserve specimen integrity.*
2. Place sample in a tissue culture plate containing 10 mL ice cold 1X PBS. Use a clean razor blade to cut a piece of tissue (around 5 mm^3) into small pieces (typically 1mm^3 or smaller) to improve cross linking efficiency.
3. Transfer tissue sample to a 50 mL tube.
4. Spin 50 mL tube at 800 x g at 4°C for 5 minutes to pellet sample then, remove supernatent.
5. Resuspend sample in 20 mL ice cold PBS and add 165 µL of 37% formaldehyde (or 330 µL of 18.5% formaldehyde) to cross link. Gently swirl tube to mix.
6. Incubate at room temperature for 15 minutes.
7. Add 2 mL of 10X glycine to quench excess formaldehyde. Mix and incubate at room temperature for 5 minutes.
8. Spin at 800 x g at 4°C for 5 minutes to pellet the sample.
9. Wash twice with 20 mL ice cold 1X PBS, then resuspend in 5mL cold 1X PBS.
10. Homogenize sample several times using a chilled Dounce homogenizer (loose pestle) on ice.
11. Transfer homogenate to a 15 mL conical tube and spin at 800 x g at 4°C for 5 minutes to pellet cells, and carefully remove supernatant.
12. Cell pellet can be snap-frozen in liquid nitrogen and stored at -80°C for future use or immediatly used for next step. If using cell pellet immediately, maintain cells on ice.

C. Cell Lysis to Release Cross-Linked Proteins/RNA

1. Prepare 0.5 mL of Nuclei Isolation Buffer containing 2.5 µL of 200X Protease Inhibitor Cocktail III and 1.25 µL of RNase inhibitor for each cell pellet (1 x10^7 cells), and keep it on ice.
    - *For tissues use 100 µL lysis buffer for every 10 mg of tissue.*
2. If necessary, thaw cell pellets that have been stored at -80°C on ice.
3. Resuspend cell pellet in Nuclei Isolation Buffer (prepared in Step 1).

4. Incubate on ice for 15 minutes: vortex the cell suspension at high speed for 10 second every 5 minutes to enhance disruption of the cell membrane.
5. (Optional) At the end of the incubation, homogenize the cell suspension 10 times in a Dounce homogenizer (loose pestle) to facilitate the release of the nuclei.
6. Spin the cell suspension at 800 x g at 4◦C for 5 minutes.
7. During 5 minute spin, prepare 0.5 mL of RIP Cross-linked Lysis Buffer containing 2.5 µL of 200X Protease Inhibitor Cocktail III and 1.25 µL of RNase inhibitor for each sample.
8. Remove supernatant. Resuspend cell (Nuclei) pellets in 0.5 mL of RIP Cross-linked Lysis Buffer containing 2.5 µL of 200X Protease Inhibitor Cocktail III and 1.25 µL of RNase inhibitor prepared in Step 7).
    - *For every 1 x 10^7 HeLa cells, 0.5 mL of RIP Cross-linked Lysis Buffer is recommended when using this protocol. It is recommended that cell concentration be less than 2 x 10^7 cells/mL, as the ratio of lysis buffer to cell density is important for reliable cell lysis.*
    - *For tissue, use same volume of RIP Cross-linked Lysis Buffer as was used for Nuclei Isolation (step 1 above).*
9. If optimal conditions for sonication have already been determined, proceed to Section D. Otherwise see Appendix A for guidelines on optimization of sonication conditions.

D. Sonication of Isolated Chromatin to Shear DNA

Important: Optimal conditions need to be determined to shear cross-linked DNA to ~200-1000 base pairs in length. See Appendix A for a typical protocol. Once shearing conditions have been optimized, proceed with the steps below.

1. If desired, remove 5 µL of the lysate from Section C, Step 8 for microfluidic electrophoresis (e.g. Agilent Bioanalyzer) or agarose gel analysis of unsheared DNA along with the shared cross-linked chromatin sample at the step 4 of this section.
2. Sonicate the lysate on wet ice (ice-water mixture).
    - *The efficiency of sonication depends upon cell type, cell equivalents and instrumentation. When possible, consult your instrument manufacturer's guidelines for instrument operation. To provide a visual reference, an example of sonicated HeLa cell chromatin suitably fractionated for use with Magna Nuclear RIP (Cross-Linked) kit is shown in Figure 8.*
    - *Keep cell lysate ice cold. Sonication produces heat, which can denature the chromatin. Allow at least 30 seconds between cycles of sonication to prevent sample overheating which can damage protein epitopes.*
3. Spin the sonicated lysate at 10,000 x g at 4°C for 10 minutes.
4. Transfer 5 µL of the supernatant (Sheared cross-linked chromatin) to a new microcentirifuge tube and place on ice. Analyze the efficiency of shearing with this sample according to the protocol in Appendix A. Steps VII to X.
    - *Store on ice if the analysis will be done in the same day; otherwise store the sample at - 80ºC.*
    - ***It is important to perform this analysis to ensure chromatin is sheared to appropriate size.***
5. Transfer 50 µL aliquots of the supernatant into new micro centrifuge tubes on ice. (up to 10 tubes)
6. Aliquots of sheared cross-linked chromatin can be snap-frozen in liquid nitrogen and stored at -80° C. If continuing on to Section E maintain samples to be used on ice.
    - *If 1 x 10^7 cells were used for chromatin preparation, each 50 µL aliquot contains ~1 x 10^6 cell equivalents of chromatin. This is sufficient for one Nuclear RIP Cross-linked reaction.*
    - *For a single set of experiment often a positive and negative antibody are utilized in each experiment, so 100 µL (or 2 vial) of sheared cross-linked chromatin can be used. Aliquoting of chromatin should be adjusted accordingly to avoid multiple freeze-thaws.*
    - *Sheared cross-linked chromatin can be stored at -80°C for up to 3 months.*
    - *Avoid additional freeze-thaw cycles to prevent protein and RNA degradation.*

E. Preparation of magnetic beads for immunoprecipitation

Key Considerations Before Starting This Section

The Nuclear RIP procedure relies on the use of high quality antibodies to perform immunoprecipitation of RNA-binding protein/RNA complexes. The amount of antibody used for immunoprecipitation will depend on the presentation (e.g. purified or unpurified) and effective affinity of the candidate antibody when used for immunoprecipitation. For purified antibodies, 1 to 5 µg per immunoprecipitation is suggested as a guideline, but the quantity may need to be optimized for antibodies from different suppliers. As a starting point the amount of antibody used should reflect that used in successful ChIP or RIP reactions performed in your lab. Our RIPAb+™ and ChIPAb+™ Validated Antibody Primer Sets have been titrated for optimal performance in RIP and ChIP and are highly recommended for use with this protocol.

When performing wash steps with magnetic beads, the use of a vacuum aspirator is recommended. To avoid introduction of RNases, use an aspirator pipette with the addition of RNase free sterile microtips when possible.

1. Label the appropriate number of 1.5 mL microcentrifuge tubes for the number of desired Nuclear RIP reactions.
   - *Label one microcentrifuge tube for each antibody of interest (user supplied) and and one negative control antibody generated from the same species as the antibody of interest.*
   - *If using the EZ-Magna Nuclear RIP Native (Cat. # 17-10523) the provided Anti-Ezh2 (Part # CS203195) and negative control Normal Mouse IgG (Part # CS200621) serve as positive and negative controls for the RIP assay*.
2. Completely disperse and re-suspend Magna ChIP Protein A/G Magnetic Beads (Part # CS207374) by end over end rotation or by pipetting. No clumps of beads should be visible
3. For each reaction planned in step 1 above, transfer 10 µL of Magna ChIP protein A/G Magnetic Beads to a microcentrifuge tube. When performing multiple reactions, prepare one additional reaction to ensure sufficient material for all assays. For example, if 5 reactions are planned transfer 60 (50+10) µL of Magna ChIP protein A/G Magnetic Beads.
4. Add five times the original bead volume of Nuclear RIP Dilution Buffer for the number of Nuclear RIP samples (50 µL Nuclear RIP Dilution Buffer per 10 µL of original volume of the magnetic beads) 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
Millipore Cat. # 20-400

5. Remove the supernatant making sure not to aspirate any magnetic beads. Remove the tubes from the magnet.
6. Repeat Step 4 and Step 5 for one additional wash.
7. Re-suspend the beads in 100 µL of the Nuclear RIP Dilution Buffer per 10 µL of original volume of magnetic beads. If multiple reactions are being performed, transfer 100 µL of the beads suspension to each microcentrifuge tube. Add ~5 µg of the antibody of interest to each microcentrifuge tube.
   - *For best results, the amount of antibody used per Nuclear RIP (Cross-linked) reaction should be experimentally determined or multiple immunoprecipitations using different amounts of antibody should be performed. For most antibodies testing a range of 1-5 µg of purified antibody is generally appropriate for a standard immunoprecipitation. If*

Removal of supernatant using Magna GrIP Rack

more than 5 µg of antibody is used, increase the volume of the magnetic beads for each reaction.

- *Use 1 µg of antibody per reaction when performing EZ-Magna Nuclear RIP (Cross-Linked) (Cat. # 17-10521) control assay with Anti-Ezh2 (Part # CS203195) and Normal Mouse IgG (Part # CS200621)*
- *It is highly recommended you perform a negative control RIP reaction using normal IgG of same species of the testing antibody.*

8. Incubate with rotation for 30 minutes at room temperature.
9. Centrifuge the tubes briefly and place on the magnetic separator for 1 minute and remove the supernatant.
10. Remove the microcentrifuge tubes from the magnet. Add 0.5 mL of Nuclear RIP Dilution Buffer to each tube and mix the beads by gently pipetting several times to completely resuspend beads. Place the microcentrifuge tube on a magnetic separator for 1 minute then remove supernatant.
11. Repeat step 10 for two additional washes. Be sure to remove final wash leaving only beads.
12. Remove the microcentrifuge tubes from the magnet and place them on ice. Close tube caps to avoid drying out of beads. These samples will be used in section G step 2. .

F. DNase I treatment of the sheared cross-linked chromatin (Optional)

Note: *DNaseI treatment of the sheared cross-linked chromatin is recommended to reduce contamination of unexpected RNAs.*

1. Rapidly thaw the sheared cross-linked chromatin (prepared in **section D**) at 37°Cand centrifuge at 10,000 x g for 10 minutes at 4°C. Carefully transfer the supernatant to new microcentrifuge tube.
2. To each 50 µL sample of sheared cross-linked chromatin add 5 µL of DNase I Supplement (Part# CS216134) and 5 µL of DNase I (2U/µL) for a final DNase I concentration of 200U/mL).
3. Incubate the tubes for 20 minutes at 37°C.
4. Add 1 µL of 0.5M EDTA (Part# CS203175) to each tube to stop reaction and vortex briefly.
5. Centrifuge at 10,000 x g for 10 minutes at 4°C.
6. Place the microcentrifuge tubes on ice until **section G** step 3.

G. Immunoprecipitation of Nuclear RNA-binding Protein-RNA complexes (Nuclear RIP)

1. Prepare the nuclear RIP Immunoprecipitation Buffer. Each immunoprecipitation requires 450 µL of Nuclear RIP Immunoprecipitation Buffer. Add 2.5 µL of Protease Inhibitor Cocktail III and 2.5 µL of RNase inhibitor to 445 µL of RIP Wash Buffer for each reaction. (Table 4.)

Component	x 1	x N
Nuclear RIP Dilution Buffer	445 µL	445 µL x_____=_____
Protease Inhibitor Cocktail III, Animal Free **Contains DMSO	2.5 µL	2.5 µL x_____=_____
RNase Inhibitor	2.5 µL	2.5 µL x_____=_____
Total	**450**	**450 µL x_____=_____**

2. Add 450 µL of RIP Immunoprecipitation Buffer to each beads-antibody tube from Step 12 section **E.** Mix by gently pipetting several times to completely resuspend beads. Place on ice.
3. If using DNase I treated chromatin, remove 50 µL of supernatant (DNase I treated sheared cross-linked chromatin) prepared in section E step 5 and add to each beads-antibody complex in Nuclear RIP Immunoprecipitation Buffer prepared above. The final volume of the immunoprecipitation reaction will be 0.5 mL. If not using DNase I treated chromatin follow step 4 below. Otherwise proceed to step 5.

4. If the sheared cross-linked chromatin was not treated with DNaseI, thaw the sheared cross-linked chromatin (From **section D**) quickly and centrifuge at 10,000 x g for 10 minutes at 4°C. Add the supernatant (untreated sheared cross-linked chromatin) to each beads-antibody complex in Nuclear RIP Immunoprecipitation Buffer.
5. Remove 5 µL of the supernatant from the remaining sheared cross-linked chromatin and place it into a new microcentrifuge tube and label "**input**". Store this input sample at -80°C until Elution of Protein/RNA Complexes and Cross-links Reversal **(section H).** This sample represents '10% input' which will be used to generate a standard curve or for comparison in RT-PCR methods.
    - *(Optional) Remove 10 µL of the supernatant of sheared cross-linked chromatin to test the expression of RNA-binding protein of interest by western blotting. Add 10 µL of 2 X SDS-PAGE loading buffer to the 10 µL of RIP lysate followed by heating at 95°C. The supernatnat can be directly applied on SDS-PAGE.*
6. Incubate all tubes with rotation for 4 hours to overnight at 4°C.
7. Centrifuge the Nuclear RIP reactions briefly to remove liquid from cap and sides of the microcentrifuge tube. Place on a magnetic separator for 1 minute.
8. Discard the supernatant, being careful not to disturb the magnetic beads.
9. Remove tubes from the magnet. Add 500 µL cold Low Salt Wash Buffer (Part# CS200625), and mix the beads by gently pipetting several times to completely resuspend beads.
10. Place tubes on a magnetic separator for 1 minute then discard supernatant.
11. Repeat the above wash procedure (steps 9 to step 10) with 500 µL of following wash buffers.
    a. Cold High Salt Wash Buffer (Part# CS200626), **one wash**
    b. Cold LiCl Wash Buffer (Part# CS200627), **one wash**
    c. Cold TE Buffer (Part# CS200628), **one wash**
        - *Optional - Remove 50 µL each out of 500 µL of the beads suspension during the last wash with TE Buffer to test the efficiency of immunoprecipitation by western blotting. The proteins can be eluted off the beads by re-suspending the beads in 1X SDS-PAGE loading buffer followed by heating at 95°C. The beads can then be centrifuged down and the supernatant directly applied on SDS-PAGE.*
12. Place the tubes on ice and immediately proceed to Elution of Protein/RNA Complexes and Cross-link Reversal (**Section H**) to recover RNA.

H. Elution of Protein/RNA Complexes and Cross-link Reversal

Prior to starting this section:
Thaw Proteinase K (Part# CS207286) and warm the RIP Elution Buffer (w/o Proteinase K, Part# CS216131) and 10% SDS (Part# CS216132) to room temperature. Ensure the 10% SDS is in solution before proceeding.

1. Prepare Nuclear RIP Elution buffer for all RIP tubes as well as Input tubes prepared in **Section G** step 5. Each Immunoprecipitate requires 200 µL of Nuclear RIP Elution buffer (178 µL of RIP Elution Buffer, 20 µL of 10% SDS, and 2.0 µL of Proteinase K (Table 3).

Table 3. Nuclear RIP Elution Buffer

Component	x 1	x N
RIP Elution Buffer	178 µL	178 µL x_____=_____
10 % SDS	20 µL	20 µL x_____=_____
Proteinase K	2.0 µL	2.0 µL x_____=_____
Total	**200 µL**	**200 µL x_____=_____**

2. Add 200 of Nuclear RIP elution Buffer prepared as described above and incubate at 60°C for 30 minutes with shaking.
    - *Shaking and 60°C incubation can be accomplished with equipment such as an Eppendorf Thermomixer® system, a Labnet Shaking incubator, or a standard roller bottle hybridization oven.*
3. Cool samples down to room temperature.
4. Centrifuge the tubes briefly and place on the magnetic separator for 1 minute.
5. Carefully transfer the supernatant to a new microcentrifuge tube and place on ice.
6. Immediately proceed to RNA Purification (**Section I**)

I. RNA Purification

Note: Make sure caps are tightly secured prior to vortexing solution in step 4.

1. Add 50 µL of Nuclease Free Water to each tube from **Section H** step 5 (total volume 250 µL).
2. Add 0.75 mL of Trizol® LS Reagent to each tube. Pipette up and down several times to mix completely.
3. Incubate all tubes for 5 minutes at room temperature.
4. Add 200 µL of chloroform to each tube. Vortex for 15 seconds and centrifuge at 12000 x g for 10 minutes at 4°C to separate the phases.
5. Remove the aqueous phase carefully and place it in a new tube. Take the same volume of upper phase for each of your samples.
6. To each tube add, Precipitate Enhancer (2 µL Pellet Paint® or 5 µL of linear acrylamide) followed by 500 µL of isopropanol. Mix and incubate at room temperature for 15 minutes to precipitate the RNA.
7. Centrifuge at 12,000 x g for 10 minutes at 4°C and remove the supernatant being careful not to disturb the pellet
8. Wash the pellet once by adding 1 mL of ice-cold 75% ethanol. Centrifuge at 12,000 x g for 5 minutes at 4°C. Carefully discard the supernatant and allow pellets to air dry.
    - *Note: Be careful when air drying RNA pellet, as over-dried pellets are difficult to resuspend.*
9. Re-suspend RNA pellet in 16 µL of Nuclease-free water, and place all samples on ice.

J. DNase I Digestion

1. Prepare 2 µL of 0.1M EDTA (Stop Solution) for each sample
2. Add 2 µL of 10X DNase I Reaction Buffer and 2 µL of DNase I to each tube from **Section I** step 9. (total volume will be 20 µL)
3. Incubate all samples for 20 minutes at 37°C
4. Centrifuge the tubes briefly and add 2 µL of Stop solution.
5. Incubate all samples for 10 minutes at 65°C
6. Centrifuge the tubes briefly and place tubes on ice.

K. Analysis of immunoprecipitated RNA

RNAs isolated using the Magna Nuclear RIP kit can be analyzed by several molecular methods including quantitative RT-PCR (if binding targets of the RBP are known), or by microarray or deep sequencing methods. Given RNA targets of known sequence, RNA specific primers can be designed that allow validation (and quantification) of the RNA immunoprecipitated by the antibodies used. Once successful Nuclear RIP can be confirmed, further interrogation of the population of RNAs in an immunoprecipitation may be pursued by population based methods such as comparative microarray

hybridization of resulting cDNAs or by deep sequencing of molecularly adapted products of the RIP reaction (see Baroni, T.E. *et al*. (2008). *Methods Mol Biol.* 419:93-108).

Presented below are illustrative methods for performing real time quantitative measurement of Nuclear RIP experiments using the control antibody supplied in the EZ-Magna Nuclear RIP native kit (Cat. # 17-10521, Anti-EZH2 Part # CS203195). Verification of Nuclear RIP enrichment can be performed using the relative standard curve method of qPCR analysis to compare RNA from a mock IP vs. RNA immunoprecipitated using your RIP antibody, or can alternatively be compared using the comparative Ct (ΔΔCt) method with two PCR amplicons, a positive control binding RNA, and a negative control binding RNA. Input RNA is required whether using relative standard curve method or the comparative Ct (ΔΔCt) method. Examples of significant enrichment are shown in Figures 2 (EZH2), 3 (SUZ12). Figure 4 shows significant fold-enrichment by the anti-EZH2 Ab of a positive control RNA (NEAT1) compared to a negative control RNA (U1) by the comparative Ct method (ΔΔCt).

L. 1-Step Real-time Quantitative RT-PCR

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 RIP sample is recommended).
   - *2.5 µL or less Nuclear RIP RNA is recommended for a 25 µL RT-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 reverse cross-linked RNA from the 10% input sample (section G, step 5), and use these samples to build a standard curve. Concentration of the Nuclear RIP samples can be calculated as percent of input using the standard curve. Alternatively, data can be calculated in relation to cell equivalents of chromatin, or mass of purified RNA, if desired.*
2. Prepare a master reaction mix as shown in Table 3. 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 3. 1-Step qRT-PCR reagent setup and running parameters

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

SYBR® Green Master Mix	12.5 µL
Reverse Transcriptase	0.5 µL
ddH_2O	9.0 µL
Primer mix	1.0 µL
Total	23 µL

qPCR parameters:

cDNA Synthesis	50°C 10 min	
Reverse Transcriptase Inactivation	95°C 5 min	
Denature	95°C 15 sec	} 40 times
Anneal and Extend:	60°C 1 min	

M. Data Analysis

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

I. Normalize DNA 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 100% input sample, perform quantitative real-time PCR with these input samples, Nuclear RIP RNA samples, and control samples (IgG, non- immunized serum, or no antibody control).
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 ChIP DNA as percent of input using the standard curve.
5. Determine the fold enrichment by calculating the ratio of $C_{Ab\ of\ interest}$ and C_{IgG}.
6. For each independent experiment, we suggest that you perform the following ChIP qPCR assays in triplicates in the same plate, if possible.
   - *For the positive control experiment, the antibody of interest is the Anti-EZH2 antibody provided in the kit, the RNAs of interest is the human long noncoding RNA NEAT1 (primers provided) and the negative control RNA is human U1 snRNA.(primers provided)*

Nuclear RIP RNA	Negative Control RNA	Positive Control RNA	RNA of Interest 1	RNA of Interest 2	RNA of Interest 3
Input dilution series 1	X	X	X	X	X
Input dilution series 2	X	X	X	X	X
Input dilution series 3	X	X	X	X	X
Input dilution series 4	X	X	X	X	X
Nuclear RIP with antibody of interest	X	X	X	X	X
Nuclear RIP with negative control antibody (IgG/NIS)	X	X	X	X	X

II. ΔΔCt method

1. Perform quantitative real-time RT-PCR with 2μL of Nuclear RIP RNA, and input RNA in triplicates.
2. Perform quantitative real-time RT-PCR with primer set targeting a positive RNA and primer set targeting a RNA separately.
3. Calculate the threshold cycle (Ct) values using real-time detection system software from qPCR equipment manufacturer.
4. Normalize Nuclear RIP RNA Ct values to input (ΔCt) for both Nuclear RIP with antibody of interest and with negative control antibody by subtracting the Ct value obtained for the input RNA from the Ct value for Nuclear RIP RNA: $\Delta Ct = Ct_{RIP} - (Ct_{input}\text{-Log2 [Input Dilution Factor]})$ (Input dilution factor is 10 if using 10% input sample).
5. Calculate the percent of input for each RIP: $\%Input = 2^{(-\Delta Ct\ [normalized\ RIP])}$.
6. Normalize Nuclear RIP with antibody of interest ΔCt values to negative control antibody (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 antibody of interest in Nuclear RIP RNA over the negative control antibody: Fold enrichment $=2^{-\Delta\Delta Ct}$.
   - *For each independent experiment, we suggest that you perform the following Nuclear RIP qRT-PCR assays in triplicates in the same plate if possible.*
   - *For the positive control experiment, the antibody of interest is the Anti-EZH2 antibody provided in the kit, the RNAs of interest is the human long noncoding RNA NEAT1 (primers provided) and the negative control RNA is human U1 snRNA.(primers provided)*

Nuclear RIP RNA	Positive Control RNA	Negative control RNA	RNA of Interest 1	RNA of Interest 2	RNA of Interest 3
Input	X	X	X	X	X
Nuclear RIP with antibody of interest	X	X	X	X	X
Nuclear RIP with negative control antibody (IgG/NIS)	X	X	X	X	X

Nuclear RIP-seq (NGS Analysis)

The Magna Nuclear RIP (Native) and (Cross-Linked) kits have been validated for RNA-seq transcript discovery using standard RNA-seq library construction products, such as the Encore® Complete RNA-Seq DR Multiplex Library System or a combination of Ovation® RNA-Seq System V2 and Encore® Rapid DR Multiplex System. 5 to 10 ng of purified RNA from Nuclear RIP reactions can be used for Ovation RNA-Seq System V2 reaction or 50 ng of purified RNA from Nuclear RIP reactions can be used for Encore Complete RNA-Seq strand-specific library construction. After the DNase I digestion (Section H of the protocol) RNA was column purified with RNeasy® Mini RNA kit (QIAGEN) before processing library construction. The analysis of Nuclear RIP RNA-Seq library can be performed between a Nuclear RIP and input RNA (total RNA), between different antibodies (protein targets), or between different stimulated condition of the cell lysates, etc. It is possible to perform analysis using the mock IgG control. However, given the low amounts of material that are recovered from this sample, amplification of the RNA may be required. Mock IgG samples tend to show a similar profile as Total RNA, but on a significantly lower read depth. Consideration must be given regarding selection of mapping techniques, as many non-coding RNAs are not annotated in transcriptome reference datasets.

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 and cell harvest

a. **Prepare 22 mL of ice-cold 1X PBS** for each 150 mm culture dish. Store on ice
b. **Cross-link the cells** by adding 165 μL of 37% formaldehyde directly to 20 mL of growth medium in a 150 mm culture dish. Swirl the dish gently to mix and incubate at room temperature for 10 minutes
c. **Prepare cell-scraping PBS in a separate tube** by adding 5 μL of 200X Protease Inhibitor Cocktail III to 1 mL of 1X PBS for each sample. Store on ice
d. **Quench the excess formaldehyde** by adding 2 mL of 10X Glycine to each culture dish. Swirl the culture plates for 5 minutes at room temperature and then place them on ice
e. **Aspirate the medium,** taking care not to disturb the cells
f. **Wash the cells twice** with 10 mL of ice-cold 1X PBS
g. **Add 1 mL of cell-scraping PBS (from step I. d)** to each culture dish
h. **Scrape the cells from each dish into a separate 1.5 mL microcentrifuge tube**
i. **Pellet the cells** by centrifuging at 800 x g for 5 minutes at 4°C
j. **Carefully remove the supernatant.** Cell pellets can be snap-frozen in liquid nitrogen and stored at -80°C or used immediately for cell lysis and sonication

II. Cell Lysis

a. **Prepare Nuclei Isolation Buffer.** For 500 μL of Nuclei Isolation Buffer, add 2.5 μL of protease inhibitor cocktail and 1.25 μL of RNase inhibitor. Keep prepared buffer on ice
b. **Thaw cross-linked cell pellets** (or use freshly prepared cell pellets from step I. i)
c. **Resuspend the cell pellet** in 500 μL of Nuclei Isolation Buffer. Mix by pipetting up and down several times until the cells have been dispersed and the mixture appears homogeneous.
d. **Incubate on ice for 15 minutes.** Vortex the cell suspension for 10 seconds at high-speed every 5 minutes to enhance cell lysis
e. **Centrifuge** the cell suspension at 800 x g for 5 minutes at 4°C
f. **Prepare RIP Cross-linked Lysis Buffer.** For 500 μL of RIP Cross-linked Lysis Buffer, add 2.5 μL of 200X Protease Inhibitor Cocktail III and 1.25 μL of RNase inhibitor. Keep buffer on ice
g. **Remove supernatant and resuspend each nuclear pellet in 500 μL of RIP Cross-linked Lysis Buffer** containing protease and RNase inhibitors**.** Mix by pipetting up and down several times until the nuclei are dispersed and the mixture appears homogeneous

III. Sonication of Isolated Chromatin to Shear DNA

a. **Sonicate the lysate on wet ice** (ice-water mix) using conditions optimized for the cells of interest
b. **Centrifuge the sonicated lysate** at 10,000 x g for 10 minutes at 4°C
c. **Transfer 50 μL aliquots of the supernatant** into fresh microcentrifuge tubes on ice. These can be snap-frozen in liquid nitrogen and stored at -80°C or used immediately for immunoprecipitation. If desired, use 5 μL of the supernatant to analyze the efficiency of shearing (Appendix A)

IV. Preparation of Magnetic Beads for Immunoprecipitation

a. **Completely disperse and resuspend Magna ChIP Protein A/G Magnetic Beads** by end-over-end rotation or by pipetting
b. **Transfer** 10 μL of Magna ChIP protein A/G Magnetic Beads to a microcentrifuge tube
c. **Wash** the magnetic beads twice with 50 μL of Nuclear RIP Dilution Buffer using a magnetic separator (i.e. Millipore Cat. # 20-400). Discard the supernatant and re-suspend the beads in 100 μL of Nuclear RIP Dilution Buffer
d. **Add ~5 μg of the antibody of interest to the tube.** Incubate with rotation for 30 minutes at room temperature. Centrifuge the tubes briefly and place on a magnetic separator for 1 minute and remove the supernatant

e. **Wash** the beads three times with 0.5 mL of Nuclear RIP Dilution Buffer. Make sure beads are completely re-suspended in the wash buffer by gentle pipetting between washes
f. **Remove the supernatant** and place the capped tubes on ice

V. DNase I treatment of the sheared cross-linked chromatin (optional)

a. **Thaw the sheared cross-linked chromatin** (from III. c above) and centrifuge at 10,000 x g for 10 minutes at 4°C. Transfer the supernatant to a new microcentrifuge tube
b. **Add 5 µL of DNase I Supplement and 5 µL of DNase I** to each tube and incubate for 20 minutes at 37°C. Add 1 µL of 0.5M EDTA to each tube to stop the reaction and vortex briefly
c. **Centrifuge** at 10,000 x g for 10 minutes at 4°C and place the tubes on ice

VI. Immunoprecipitation

a. **Prepare Nuclear RIP Immunoprecipitation Buffer.** For 450 µL of Nuclear RIP Immunoprecipitation Buffer, add 2.5 µL of RNase inhibitor and 2.5 µL of Protease Inhibitor Cocktail III to 445 µL of RIP Wash Buffer
b. **Add 450 µL of RIP Immunoprecipitation Buffer to each microcentrifuge tube** containing the antibody bound to the magnetic beads. Mix by gently pipetting several times to completely resuspend the beads. Place the tubes on ice
c. **Add 50 µL of the DNase I-treated sheared cross-linked chromatin** (from step V. c) to each magnetic bead-antibody complex. If the sheared chromatin was not treated with DNase I (step III. C), quickly thaw the sample, centrifuge at 10,000 x g for 10 minutes at 4°C, and add 50 µL of the supernatant to the bead-antibody complex
d. **Store 5 µL of the remaining sheared cross-linked chromatin** in a separate tube at -80°C for use as "input". This represents "10% input" and will be used to generate a standard curve or for comparison in RT-PCR analysis
e. **Incubate the microcentrifuge tubes at 4°C** on a rotating rack for 4-16 hours or overnight
f. **Centrifuge the tubes** briefly to remove liquid from the cap and sides of the microcentrifuge tube
g. **Place the tubes on a magnetic separator for 1 minute.** Remove and discard the supernatant without disturbing the beads
h. **Add 500 µL of ice-cold Low Salt Wash Buffer** to each tube after removing them from the magnetic separator. Gently pipette to completely resuspend the beads
i. **Place the tubes on a magnetic separator for 1 minute.** Remove and discard the supernatant without disturbing the beads
j. **Wash the beads one more time** with 500 µL of ice-cold Low Salt Wash Buffer
k. **Wash the beads once with ice-cold LiCl Wash Buffer**
l. **Wash the beads once with ice-cold TE Buffer**
m. **Place the tubes on ice and immediately proceed to cross link reversal and RNA elution**

VII. Cross-link Reversal

a. **Thaw Proteinase K and warm the RIP Elution Buffer to room temperature to ensure the SDS is in solution**
b. **Prepare 200 µL of Nuclear RIP Elution Buffer** by adding 20 µL of 10% SDS and 2 µL of Proteinase K to 178 µL of RIP Elution Buffer
c. **Add 200 µL of Nuclear RIP Elution Buffer to the washed magnetic beads** (Step VI. m) and incubate in a shaking incubator for 30 minutes at 60°C
d. **Cool the samples** down to room temperature, centrifuge briefly, and place on a magnetic separator for 1 minute
e. **Carefully transfer the supernatant to a fresh tube,** place on ice and immediately process for RNA purification

VIII. RNA Purification

a. **Add 50 μL of Nuclease-Free Water** to each tube from the previous step (VII. e)
b. **Add 0.75 mL of Trizol®** to each microcentrifuge tube from the previous step. Pipette gently to resuspend the magnetic beads
c. **Thaw the input sample (10 μL, step IV. d)** and add 1.0 mL of Trizol®. Mix well
d. **Incubate all the tubes at room temperature for 5 minutes**
e. **Add 200 μL of chloroform** to each tube. Cap the tubes tightly and vortex for 15 seconds. Centrifuge at 12,000 x g for 10 minutes at 4°C
f. **Transfer the same volume of aqueous phase** from each sample to a new tube. Add a suitable carrier, followed by 500 μL of isopropanol. Incubate at room temperature for 15 minutes to precipitate the RNA
g. **Centrifuge at 12,000 x g for 10 minutes at 4°C** and discard the supernatant carefully
h. **Wash the pellet with 1 mL of ice-cold 75% ethanol.** Centrifuge at 12,000 x g for 5 minutes at 4°C and discard the supernatant. Air-dry the pellets
i. **Resuspend the pellets** in 16 μL of Nuclease-free water and place the tubes on ice

IX. DNase I Digestion

a. **Prepare 2 μL of 0.1M EDTA** for each sample
b. **Add 2 μL of 10x DNase I Reaction Buffer and 2 μL of DNase I** to each sample
c. **Incubate the samples for 20 minutes at 37°C**
d. **Centrifuge the tubes briefly and add 2 μL of 0.1M EDTA to each tube**
e. **Incubate the samples at 65°C for 10 minutes**
f. **Centrifuge the tubes briefly and place on ice**
g. **The samples are now ready for analysis**

Figure 2: Performance of Magna Nuclear RIP Cross-Linked Kit, EZH2

Nuclear RIP was performed using HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1 (Positive target, Part # CS216139)and U1snRNA (Negative Target, Part # CS203215).

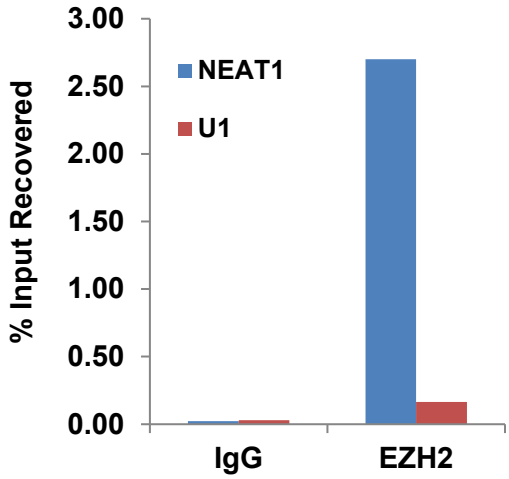


Figure 3 Performance of Magna Nuclear RIP Cross-Linked Kit, SUZ12

Nuclear RIP was performed using HeLa cell lysate and either anti-SUZ12 (Cat. # 03-179) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1 (Positive target, Part # CS216139)and U1snRNA (Negative Target, Part # CS203215).

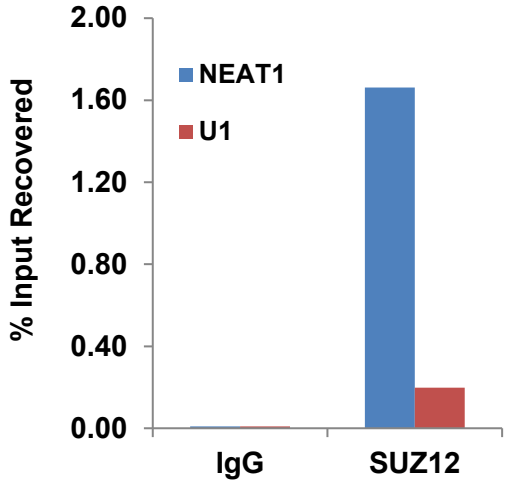


Figure 4: Association of mRNA from Cross-Linked Chromatin to EZH2

Nuclear RIP was performed using HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1, U1 snRNA and GAPDH

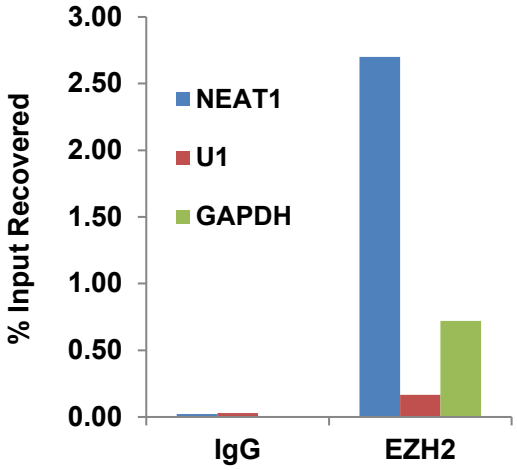
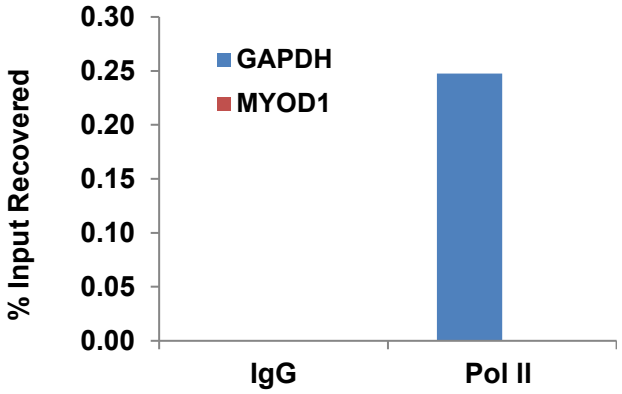


Figure 5: Performance of Magna Nuclear RIP Kit

Nuclear RIP was performed using HeLa cell lysate and either anti-RNA Polymerase II (Cat. # 17-620) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for GAPDH (Positive target)and MYOD1 (Negative Target). MYOD1 signals were lower than detectable level.



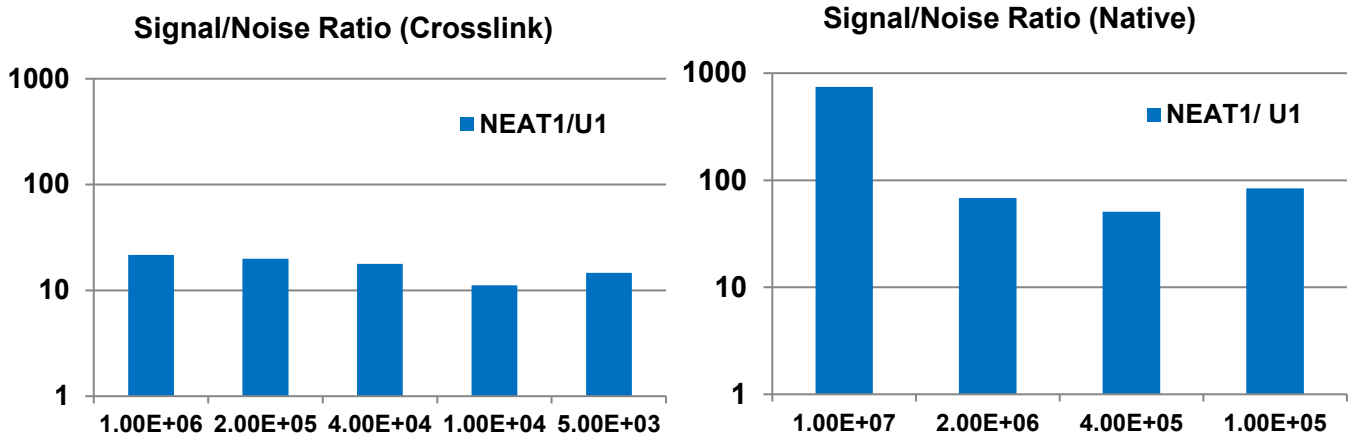


Figure 6: Comparison Cross-link with Method on Signal to Noise Ratio. Nuclear RIP was performed with (Native) and (Cross-link) kits using various amounts of HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1 (Positive target, Part # CS216139) and U1snRNA (Negative Target, Part # CS203215). Signal to noise ratio was calculated by dividing the % recovery of NEAT1 by % recovery of U1 snRNA. With native protocol, overall signal to noise ratio was higher, although cross-linking protocol retains ratio with fewer cell equivalents of chromatin.

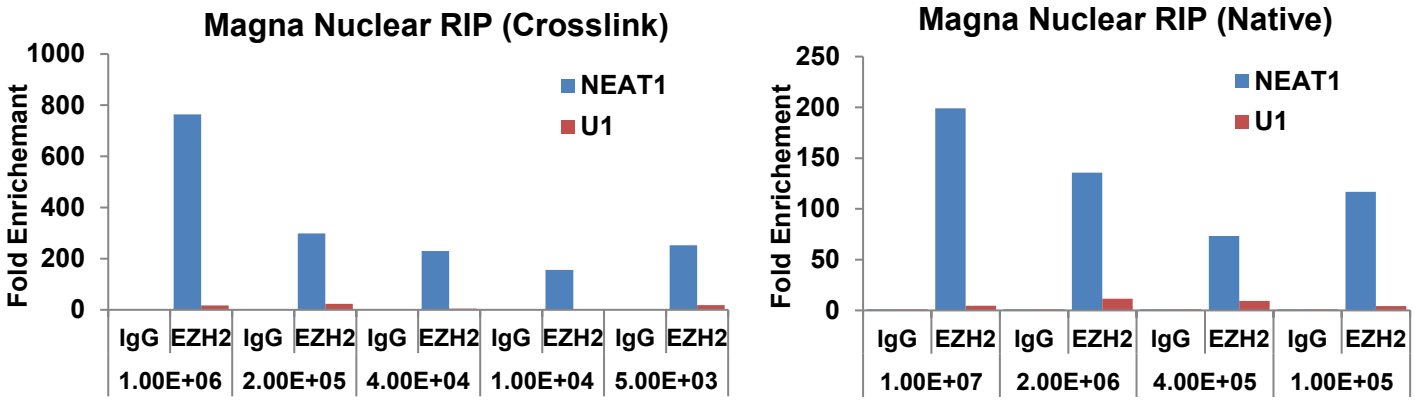


Figure 7: Comparison Cross-link with Native Method on Sensitivity. Nuclear RIP was performed with (Native) and (Cross-link) kits using various amounts of HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1 (Positive target, Part # CS216139) and U1snRNA (Negative Target, Part # CS203215). Fold enrichment over the Normal Mouse IgG was calculated by $\Delta\Delta Ct$ method. With cross-link protocol NEAT1 enrichment was shown with 5,000 HeLa Cells while with native methods required more cells.

Appendix A: Optimization of DNA Sonication

Optimal conditions for shearing cross-linked DNA to 200-1000 base pairs in length depend on the cell type, cell concentration, and the specific sonicator equipment, including the power settings and duration and number of pulses. Approaches for optimizing sonication may include the following:

A. Varying the concentration of cell equivalents per mL of RIP Cross-Linked Lysis Buffer with constant sonication parameters.
B. Choosing a fixed concentration of cell equivalents per mL of RIP Cross-Linked Lysis Buffer and varying cycles and/or power settings of sonication.
C. A combination of both approaches.

The protocol presented below describes optimization following option B and is provided as an example only.

I. Generate a cell lysate by following Section A and B.
II. Continue following the Cell Lysis procedure (section C) through Step 8. Each microcentrifuge tube should contain approximately 10^7 cells in 500 µL of cell lysate.
III. Be sure to keep the samples on wet ice at all times. Sonication generates heat which will denature the chromatin.
IV. Remove 5 µL cell lysate prior to sonication for analysis of unsheared DNA.
V. Shear the chromatin with 1, to 8 pulses at 60% power using a using a Misonix 3000 instrument and a #419 microtip probe, use 15 second pulses with 50 second intervals between pulses. Keep tubes cool at all times.
VI. Remove 5 µL sonicated chromatin from each condition to a fresh tube.
VII. To all samples (unsheared and sheared), add 40 µL RIP Elution Buffer.
VIII. Add 5 µL of 10% SDS and incubate at 65°C for 2 hours for reverse the cross-links.
IX. Cool the samples down to room temperature.
X. Centrifuge the tubes briefly.
XI. Add 50 µL of TE to each tube of cross-link reversed chromatin sample.
XII. Add 2 µL of 10 mg/mL RNaseA to each sample.
XIII. Mix and incubate at 37°C for 2 hours.
XIV. Centrifuge the tubes briefly.
XV. Add 2 µL of 10 mg/mL Proteinase K to each sample.
XVI. Mix and incubate at 55°C for 2 hours.
XVII. Cool the samples down to room temperature.
XVIII. Centrifuge the tubes briefly.
XIX. Add 200 µL of Nuclease free water to each sample.
XX. Spin down gel in Phase Lock tubes for 30 sec at 14,000 rpm.
XXI. Put samples in gel phase lock tube. Add 400 µL of buffer saturated phenol and carefully mix the sample by inverting the tube for several times. Spin for 5 min at 14,000 rpm (phenol goes below phase lock gel).
XXII. Add 400 µL of chloroform/isoamyl alcohol and carefully mix by inverting the tube several times. Spin for 5 min @ 14,000 rpm, transfer the top (aqueous) layer to a new tube.
XXIII. Add 16 µL of 5M NaCl, 1.5 µL of 1µL of glycoblue and 880 µL of 100% chilled EtOH. Mix well by inverting tube several times.

XXIV. Incubate at -80°C for 30 minutes.

XXV. Spin down the sample at maximum speed in a microcentrifuge (>14,000 rpm) for 15 minutes at 4°C.

XXVI. Wash the pellets with 500 µL of chilled 70% EtOH. Spin at 14,000 rpm for 5 minutes at 4°C.

XXVII. Air dry pellet for 10 minutes (pellet will turn clear) and resuspend pellet in 10 µL of TE buffer.

XXVIII. Determine concentration and yield.

XXIX. Run on gel (500 ng to 1 µg, depending on gel) and/or BioAnalyzer (100 ng).

XXX. Analyzed Load 10 µL on a 1-2% agarose gel.

XXXI. Observe which of the shearing conditions gives a smear of DNA in the range of 200 -1000 bp. See Figure 3 for an example.

XXXII. Repeat optimization of the shearing conditions if the results indicate that the resulting DNA is not in the desired size range. Increase the duration or the number of pulses if the chromatin DNA is too large.

DNA Sonication: Sheared DNA Should Be Between 200-1000 bp in Length

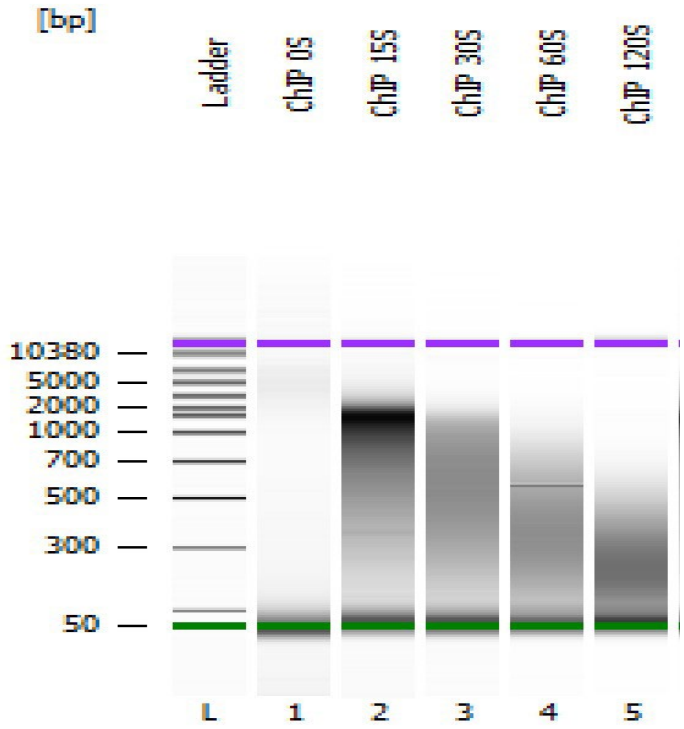


Figure 8: Figure 1. Chromatin Preparation

HeLa cells were cross-linked with 0.3% formaldehyde for 10 minutes.

Nuclei were extracted and lysed in RIP Cross-Linked Lysis Buffer. Time course experiments were performed with a probe sonicator. DNA was extracted and analyzed by Bioanalyzer.

Chromatin sonicated for 30 or 60 seconds (Lane 3 and Lane 4) demonstrated the best signals for nuclear RIP experiments.

Nuclear RIP (Cross-Linked) Optimization and Troubleshooting

Step	Potential Problems	Experimental Suggestions
Cross-linking	Not enough or too much cross-linking	The amount of formaldehyde and time of cross-linking must be determined empirically. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.
Cell Lysis	Inefficient disruption of cells	It is important to have sufficient Nuclei Isolation Buffer for the number of cells processed. Follow the guidelines in this protocol. Also, verify cell lysis by viewing a 10 µL portion of the cell lysate under the microscope to confirm lack of intact cells.
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 the appropriate size fragments.
	Denaturation of proteins from overheating sample	Keep the sample on ice during sonication. Shorten the time of each sonication and increase the number of times the sample is sonicated. Allow sufficient time for sample to cool between pulses.
Immuno-precipitation	Antibody doesn't immunoprecipitate protein in the RIP lysate	• Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis • Choose an antibody directed to a different epitope of the antigen. • Use Millipore ChIPAb+ or RIPAb+ validated antibodies where possible • Perform IP from a dilution series of antibody with a fixed amount of RIP lysate or vice versa. • Increase incubation time of the antibody of interest with the RIP lysate to overnight at 4°C. • Confirm antibody isotype is compatible with immunoprecipitation by Protein A or G. This kit is not recommended for use of IgM or chicken IgY antibodies.
	Insufficient quantity of magnetic beads in immunoprecipitation	• The magnetic beads settle to the bottom of the tube over time. Make sure the magnetic beads are well mixed prior to removing the appropriate volume for IP. • Carefully aspirate beads when using vacuum aspirator and use a high strength neodymium magnetic rack such as the Millipore Cat. # 20-400 MagnaGrIP Rack.
Washing	High background due to insufficient washing	Increase number of washes for each wash buffer.
	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 Reversal of cross-links	Incomplete elution	When performing elution, make sure that the temperature is near 60°C. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.
	Excessive Cross-linking	Excessive cross-linking may not be reversible. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.

Step	Potential Problems	Experimental Suggestions
RNA Purification	Contamination of Proteins	• Avoid the interphase when extracting RNA using Trizol® extractions.
	Low RNA yield	• Most RNA-binding protein immunoprecipitations do not yield measureable amounts of RNA. Sub nanogram quantities of RNAs can however be detected by RT-PCR. • If RNAs are not detectable following cDNA synthesis, consider Immunoprecipitation troubleshooting above.
	RNA degraded	• Use RNAse inhibitor in solutions as recommended in this protocol. Make certain RNAse free work conditions exist and RNAses are not being introduced. • Follow the guideline for the RNase control at the beginning of the protocol • Use RNAse inactivating reagents to ensure work area and materials are RNAse free
	No RNA detected	• Increase incubation time for the ethanol precipitation at -80°C. • The RNA ethanol precipitates are sometimes very small. Be sure not to suck up RNA precipitate when removing the supernatant. • Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis.
RT-PCR	No PCR product from Positive Control RIP samples	• Increase in varying amounts the RNA added to the RT-PCR reaction up to 10% of the PCR reaction if it was added less. • 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 antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis
	High background level negative control RIP samples	• Insufficient wash after immunoprecipitation. Increase the times to wash the beads. More stringent washing may be achieved by adding optimally determined concentration of sodium chloride, SDS, deoxycholate or chaotropic agents like urea (1~3M). However further caution should be taken to make sure that the antibody or target RBP are not affected by harsh washing conditions.

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