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

Nuclear RIP (Native) Kit (Catalog No. 17-10522)
EZ-Nuclear RIP (Native) Kit (Catalog No. 17-10523)

24 reactions

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Italy: 848.845.645
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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 24 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.

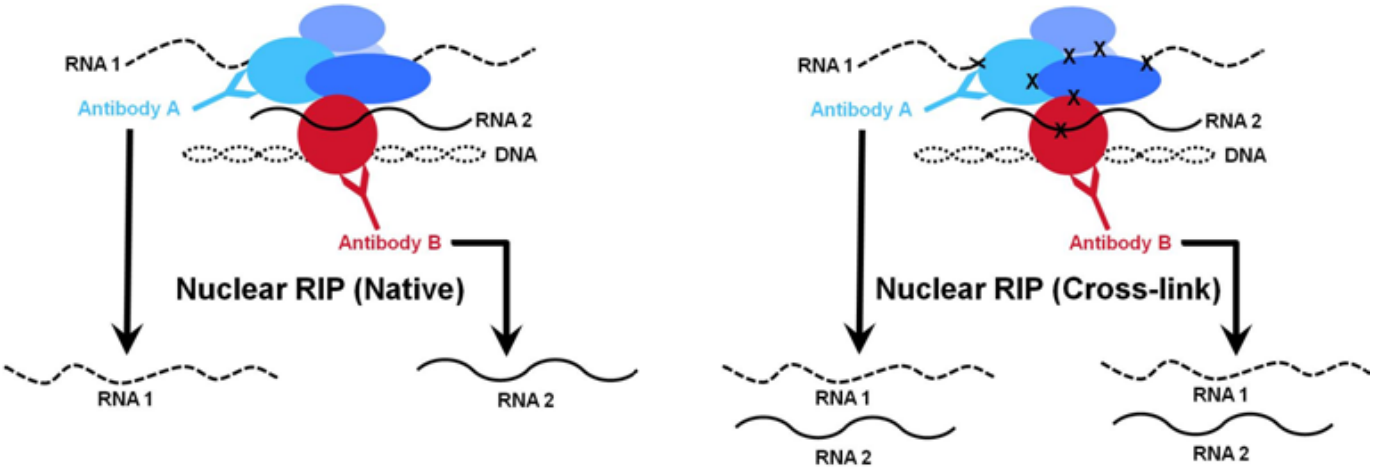

Figure 1. Mapping Protein:RNA interactions using Magna Nuclear RIP (Native) vs. Magna Nuclear RIP (Cross Link). Chromatin is prepared as the substrate for immunoprecipitation in both methods, although the details of the isolation and fragmentation procedure differ. Native RIP is expected to recover high 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-link) 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-link)		
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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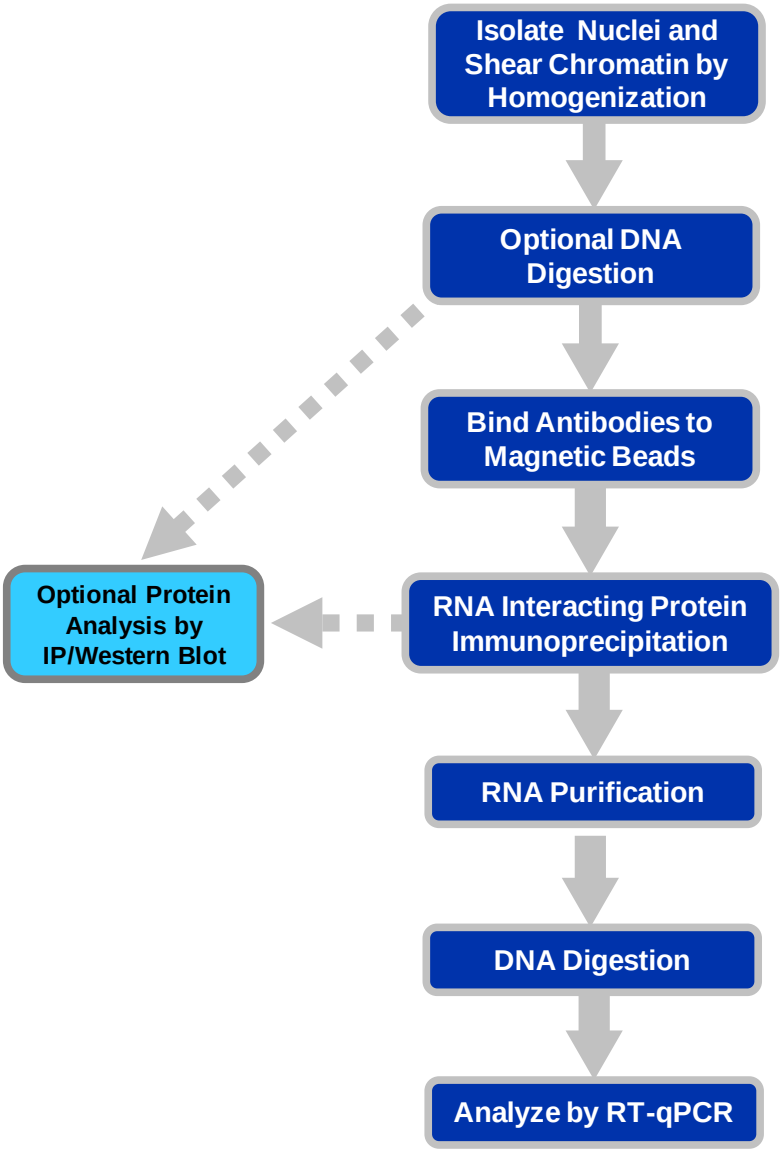
Magna Nuclear RIP Procedure Overview



Time Required : ~1.5 hrs
Note: Lysate may be stored for 3 months at -80°C)

Time Required : ~0.5 hrs

Time Required: ~1 hr
Note: Beads may be stored at 4°C for several weeks with 0.02% sodium azide

Time Required: ~4 hrs to Overnight

Time Required: ~1 hrs
Note: RNA may be stored for several weeks in ethanol at -80°C)

Time Required: ~0.5 hrs

Time Required: ~4-6 hrs
Note analysis can also be done by RNA-seq

Warnings and Precautions

***This protocol utilizes Trizol®, in which phenol is used. Contact with phenol can cause burns and can be fatal. Use gloves and other personal protective equipment when working with phenol, and assure that phenol waste is disposed of properly and in accordance with your institution's policy for disposing of organic waste.

**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.

Materials Provided (Kit Configurations)

The Magna Nuclear RIP (Native) kit provides sufficient reagents for 12 individual chromatin preparations and 24 Nuclear RNA immunoprecipitations. The EZ-Magna Nuclear RIP (Native) 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.

MAGNARIP07 (Component box of all MAGNA Nuclear RIP Native Kits) (Store at 4°C)		
Component	**Part #**	**Quantity**
Magna ChIP™ Protein A/G Magnetic Beads*	CS207374	250 µL
Nuclei Isolation Buffer	CS216140	12 mL
Nuclear RIP Wash Buffer	CS216141	150 mL
RIP Lysis Buffer	CS203176	2.4 mL
0.5M EDTA	CS203175	500 µL

MAGNARIP08 (Component box of all MAGNA Nuclear RIP Native kits) (Store at -20°C)		
DNase I (RNase Free) 2 U/µL	CS216142	320 µL
DNase I Reaction Buffer	CS216135	75 µL
Protease Inhibitor Cocktail III, Animal Free **Contains DMSO	535140-1ML	180 µL
RNAse Inhibitor	CS216144	200 µL

MAGNARIP06 (Component box of 17-10523 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) Sufficient for 10 Immunoprecipitations
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 (5 µM of each control primer)
U1snRNA 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 (5 µM of each control primer)

**The magnetic beads described here are the same as those used in Magna ChIP A/G kits (see page 24). Magna ChIP A/G beads are validated for both ChIP and RIP protocols.*

Materials Required But Not Supplied

Reagents

- Cells or tissue, 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® Reagent Life Technologies Cat. #15596-026) or similar organic extraction reagent
- Chloroform (e.g. Fisher, Cat. # BP1145)
- Isopropanol (molecular biology grade)
- 100% Ethanol (molecular biology grade)
- Carriers for RNA Precipitation
  - Pellet Paint® Co-Precipitant (125 reactions, EMD Millipore Cat. # 69049) or or RNase-free glycogen
  - If samples are intended for Next Gen Sequence library preparation, use Linear Acrylamide (5mg/mL, Life Technologies Cat. # AM9520)
- Nuclease Free Water
- 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 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

- Syringes (1 mL)
- 27G Needles
- (Dounce homogenizer (loose pestle, necessary for tissue samples but optional for cultured cells)
- Magnetic Separation Stand (e.g. Magna GrIP™ Rack 8 well, (Cat. # 20-400) or PureProteome™ Magnetic Stand, (Cat. # LSKMAGS08)
- Vacuum Aspirator
- Vortex mixer
- Rotating wheel/platform
- Centrifuge for cell culture
- Microcentrifuge capable of up to 12,000 x g
- Ultra low temperature freezer (below -80°C)
- Variable temperature water bath or incubator
- Rotating microtube mixer
- Timer
- Pipettes (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 plate, 0.2 mL

Detailed Protocol

Please read through the entire protocol and carefully plan your work before starting. The protocol below covers required steps to produce chromatin for a Nuclear RIP (native) experiment. This protocol is optimized for native sheared chromatin. If cross linked starting material is preferred, use the Magna™ Nuclear RIP (Cross Linked) kit (Cat. # 17-10520) or EZ- Magna™ Nuclear RIP (Native) kit (Cat. # 17-10521). The nuclear RIP method requires multiple steps and can be completed 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 RIP Experiments

- Calculate the number of desired immunoprecipitations. Samples include the antibodies of interest and a negative control IgG derived from 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 (Native) kit (Cat. # 17-10523).
- It is important to get a concentrated nuclear lysate for the immunoprecipitation to be successful. Typically one nuclear RIP (Native) reaction (i.e. one immunoprecipitation using one antibody) requires 100 µL of nuclear RIP lysate from ~1.0 x 10^7 cells or one 15 cm plate**.** The calculation for the volume of nuclear RIP 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, it is suggested that ~1.0 x 10^7 cells 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 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 Lysis Buffer (µL)
T-75	75	~0.5 x 10^7	250	50
T-225	225	~1.3 x 10^7	650	130
10 cm plate	78.5	~0.5 x 10^7	250	50
15 cm plate	176.6	~1.0 x 10^7	500	100

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 # 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.

B. Sample Preparation (Cultured Cells or Fresh Tissue)

I. Cultured Cells (Adherent or Suspension)

1. Stimulate or treat, if necessary, adherent mammalian cells at ~80-90% confluency in a 150 mm culture dish containing 20 mL of growth media. If using suspension cells, stimulate or treat if necessary, then spin down cells at 1,350 X g for 5 min.
    - *For HeLa cells, this is approximately 1 x 10^7 cells. This typically generates a preparation of nuclear lysate sufficient for one immunoprecipitation.*
    - *The volume of buffers supplied in the kit is sufficient to generate nuclear lysate from up to 24 x 15 cm plates of cultured cells.*
    - *Cell numbers can be scaled according to the performance of the antibody of interest to optimize signal-to-noise ratio relative to negative control (mock IgG or negative-location control). For example, Anti- Ezh2 (Part # CS203195) can perform successful RIP of as few as 1 X 10^5 HeLa cells. For simplicity this protocol is written using 1 X 10^7 cells per RIP to ensure optimal performance of the control antibodies.*
    - *Nuclei from other types of culture vessels can be isolated with slight modifications to the protocol (see table 1).*
2. Aspirate media, removing as much medium as possible, being careful not to disturb the cells or cell pellet.
3. Add 10 mL of cold 1X PBS to wash cells.
4. Carefully remove 1X PBS and repeat wash.
    - *If using suspension cells, spin down cells at 1,350 X g for 5 min. carefully aspirate media and repeat wash.*
    - *Be sure to rinse the cells twice. After second rinse, cell pellets can be snap-frozen or you can continue on to cell lysis.*
5. Add 1 mL of ice cold PBS. Scrape cells off from each flask or plate and transfer to a separate 1.5 mL centrifuge tube. If using suspension cells resuspend cell pellet in 1 mL ice cold PBS. Count cells if desired.
6. Collect cells by centrifugation at 800 x g at 4°C for 5 minutes to pellet.
7. 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 you can continue on to cell lysis. If cell pellet is frozen, thaw on ice before proceeding to Section C.

II. Fresh tissue

1. Dissect 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 1 nuclear RIP reaction. 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, on ice. 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 lysis efficiency.
3. Transfer tissue sample to a 50 mL tube on ice.

4. Spin at 800 x g at 4°C for 5 minutes to pellet the sample.
5. Wash twice with 20 mL ice cold 1X PBS, then resuspend the sample in 5mL cold 1X PBS.
6. Homogenize sample several times using a chilled Dounce homogenizer (loose pestle) on ice.
7. 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.
8. Cell pellet can be frozen and stored at -80°C for future use or immediatly used for next step. If using cell pellet immediately, maintain cells on ice,

C. Nuclear Lysate Preparation

1. Prepare an appropriate amount of complete Nuclei Isolation Buffer for the quantity of cells being lysed (see Table 2). For 500 µL of Nuclei Isolation Buffer, add 2.5 µL of protease inhibitor cocktail and 1.25 µL of RNase inhibitor and keep it on ice.
   - *For every 1 x 10^7 HeLa cells, 500 µL of Complete Nuclei isolation Buffer is recommended when using this protocol.*
   - *For tissue use 500 µL Complete Nuclei isolation buffer for every 50 mg of tissue.*

Table 2. Complete Nuclei Isolation Buffer

#	Component	x 1	x N Samples
1	Nuclei Isolation Buffer	0.5 mL	0.5 mL x_____=_____
2	Protease Inhibitor Cocktail III	2.5 µL	2.5 µL x_____=_____
3	RNase Inhibitor	1.25 µL	1.25 µL x_____=_____

2. Prepare an appropriate amount of Complete RIP Lysis Buffer for the quantity of cells being harvested (see Table 2). For 100 µL of RIP Lysis Buffer, add 0.5 µL of protease inhibitor cocktail and 0.25 µL of RNase inhibitor and keep it on ice.
   - *For every 1 x 10^7 HeLa cells, 100 µL of Complete RIP Lysis Buffer is recommended when using this protocol.*
   - *For tissue use 100 µL Complete RIP lysis buffer for every 50 mg of tissue.*

Table 3. Complete RIP Lysis Buffer

#	Component	x 1	x N Samples
1	RIP Lysis Buffer	100 µL	100 µL x_____=_____
2	Protease Inhibitor Cocktail	0.5 µL	0.5 µL x_____=_____
3	RNase Inhibitor	0.25 µL	0.25 µL x_____=_____

3. If necessary, thaw cell pellets that have been stored at -80°C on ice.
4. Resuspend cell pellets in ice cold Complete Nuclei Isolation Buffer (prepared in Step 1). Mix by pipetting up and down until the cells have been dispersed and the mixture appears homogeneous.
5. Incubate on ice for 15 minutes: vortex the cell suspension at high speed for 10 seconds every 5 minutes to enhance disruption of the cell membrane.
6. (Optional) At the end of the incubation, homogenize the cell suspension 10 times in a Dounce homogenizer on ice (loose pestle) to facilitate the release of the nuclei.
7. Spin the cell suspension at 800 x g at 4°C for 5 minutes.

8. Remove supernatant. Resuspend cell pellets in Complete RIP Lysis Buffer (Prepared in Step 2). Mix by pipetting up and down until the nuclei have been dispersed and the mixture appears homogeneous. Incubate the lysate on ice for 5 min.
9. Pass the nuclei 4 times through a 27-guage needle and place in 1.5 mL microcentrifuge tube then freeze at -80°C (snap-freeze with liquid nitrogen or dry ice/ethanol bath). These samples will be used in section E.
    - *While the volume of the amount dispensed is not critical, the volume of lysate per antibody is generally 100 µL per RIP and often a positive and negative antibody are utilized in each experiment, so 200 µL (or 2 vial) of cell lysate correlates to a single set of experiment. Aliquoting of lysate should be adjusted accordingly to avoid multiple freeze-thaws for large scale lysate preparations.*
    - *This method employs a single freeze-thaw to gently lyse the nuclei.*
    - *Immediate initial freezing of the lysate is essential to complete the lysis process.*
    - *Lysate may be stored up to 3 months at -80°C.*
    - *Avoid additional freeze-thaw cycles to prevent protein and RNA degradation.*

D. 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. Always maintain the RIP Wash Buffer on ice.

1. Appropriately label a set of of 1.5 mL microcentrifuge tubes for the number of planned 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 up and down multiple times. 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 per Nuclear RIP reaction 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.

Magna GrIP™ Rack
Millipore Cat. # 20-400

4. Add five times the original magnetic bead volume of Nuclear RIP Wash Buffer as appropriate for the number of Nuclear RIP samples (50 µL Nuclear RIP Wash buffer per 10 µL of original volume of magnetic beads) and mix by gently pipetting up and down several times to completely resuspend magnetic beads. Place the microcentrifuge tube on the magnetic separator (e.g. Millipore Cat. # 20-400) for 1 minute.
5. Remove the supernatant making sure not to aspirate any magnetic beads. Remove the microcentrifuge tubes from the magnet.
6. Repeat Step 4 and Step 5 for one additional wash.
7. Re-suspend the magnetic beads in 100 µL of the Nuclear RIP Wash 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 (Native) 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.*
    - *Use 5 µg of antibody per reaction when performing EZ-Magna Nuclear RIP (Native) (Cat. # 17-10523) 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.*

Removal of supernatant using Magna GrIP Rack

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

E. DNase I treatment of the Nuclei lysate

1. Rapidly thaw the nuclear lysate (prepared in **section C**) at 37°C and centrifuge at 10,000 x g for 10 minutes at 4°C. Carefully transfer the supernatant to new microcentrifuge tube.
2. Add 10 µL of DNase I (2 U/µL) (Part #CS216142) to each 100 µL microcentrifuge tube of nuclear lysate for a final DNase I concentration of 200 U/mL.
3. Incubate for 20 minutes at 37°C.
4. Centrifuge at 10,000 x g for 10 minutes at 4°C.
5. Place microcentrifuge tubes on ice until **section F** step 3.

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

1. Prepare the nuclear RIP Immunoprecipitation Buffer. Each immunoprecipitation requires 900 µL of Nuclear RIP Immunoprecipitation Buffer. Add 5 µL RNase inhibitor to 895 µL of RIP Wash Buffer for each reaction. (Table 4.)

Table 4. Nuclear RIP Immunoprecipitation Buffer

Component	x 1	x N Samples
Nuclear RIP Wash Buffer	895 µL	895 µL x_____=_____
RNase Inhibitor	5 µL	5 µL x_____=_____
Total	900 µL	900 µL x_____=_____

2. Add 900 µL of RIP Immunoprecipitation Buffer to each microcentrifuge tube containing antibody and magnetic beads prepared in section **D** Step 12. Mix by gently pipetting several times to completely resuspend the magnetic beads. Place the microcentrifuge tube on ice.
3. Take the centrifuged DNase I treated nuclear lysate prepared in section E step 5 and collect 100 µL of supernatant for each nuclear RIP reaction. Do not disturb the pelleted debris at the bottom of the tube. The pellet may not visible. Add the lysate to each vial of magnetic bead-antibody complex prepared in step 2 above. The final volume of the immunoprecipitation reaction will be 1.0 mL.

4. Remove 10 µL of the remaining supernatant from the remaining centrifuged DNase I treated nuclear lysate from step 3 above and place it into a new microcentrifuge tube and label "**input**". Store this sample at -80°C until the RNA purification is started (section **G**). This sample represents '10% input' which will be used to generate a standard curve or for comparison in RT-PCR methods (real-time or end-point). This input RNA sample can also be used for the optional RNA quality assessment (section **V**).
    - *(Optional) Remove 10 µL of the supernatant of nuclei lysate 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 for 5 minutes. The nuclear lysate can be directly applied on SDS-PAGE.*
5. Place the microcentrifuge tubes on a rotating rack and incubate at 4°C for 4-16 hours or overnight.
6. 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.
7. Discard the supernatant, being careful not to disturb the magnetic beads.
8. Remove the microcentrifuge tubes from the magnet. Add 500 µL ice cold Nuclear RIP Wash buffer. Mix by gently pipetting several times to completely resuspend the magnetic beads.
9. Place the microcentrifuge tubes on a magnetic separator for 1 minute and discard the supernatant.
10. Repeat steps 8 and 9 five more times to wash the magnetic beads a total of six times with 500 µL of ice cold Nuclear RIP Wash Buffer.
    - *(Optional) Remove 50 µL each out of 500 µL of the magnetic bead suspension during the last wash to test the efficiency of immunoprecipitation by western blotting. The proteins can be eluted by re-suspending the magnetic beads in 1X SDS-PAGE loading buffer followed by heating at 95°C. The magnetic beads can be removed by centrifugation and the supernatant directly applied on SDS-PAGE.*
11. After discarding the supernatant from the final wash place the magnetic beads (with the last wash removed) on ice and immediately proceed to RNA purification. (**Section G**)
    - *Process to RNA purification immediately to avoid drying of beads.*

G. RNA Purification

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

1. Add 1.0 mL of Trizol® to each microcentrifuge tube from **Section F,** Step 11. Pipette up and down several times to completely resuspend the magnetic beads.
2. Thaw the input sample (10 µL) from **section F,** step 5 and add 1.0 mL of Trizol to each tube. Pipette up and down several times to mix well.
3. Incubate all tubes at room temperature for 5 minutes.
4. Add 200 µL of chloroform to each tube. Vortex for 15 seconds and centrifuge at 12,000 x g for 10 minutes at 4°C to separate the phases.
5. Remove the upper aqueous phase carefully and place it in a new tube. Avoid withdrawing any of the interphase or organic layer. The aqueous phase should be from 400 to 500 µL. Take the same volume of upper aqueous phase for each of your samples.
6. To each tube, add carrier to enhance precipitation (2 µL of 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 carefully 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. Discard the supernatant carefully 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 in 16 µL of Nuclease-free water and place the tubes on ice.

H. DNaseI Digestion

1. Prepare appropriate amount of 0.1M EDTA by diluting 0.5M EDTA with Nuclease Free Water in a new microcentrifuge tube. Each sample (including Input samples) requires 2 µL of 0.1M EDTA at step 4 of this section.
2. Add 2 µL of 10X DNase I Reaction Buffer (Part # CS216135) and 2 µL of DNase I (Part # CS216142) to each tube from Section G step 9 (total volume will be 20 µL).
3. Incubate all samples for 20 minutes at 37°C.
4. Centrifuge microcentrifuge tubes briefly and add 2 µL of 0.1M EDTA to each tube.
5. Incubate all samples for 10 minutes at 65°C.
6. Centrifuge the tubes briefly and place tubes on ice.

I. 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.[4]

Presented below are illustrative methods for performing real time quantitative measurement of RIP experiments using the control antibody supplied in the EZ-Magna Nuclear RIP native kit (Cat. # 17-10523), 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 Figure 2 (anti-EZH2), and Figure 3 (anti-SUZ12). Figure 4 shows significant fold-enrichment by anti-EZH2 of a positive control RNA (NEAT1) compared to a negative control RNA (U1) by the comparative Ct method (ΔΔCt). Note that research into proteins in the PRC2 complex show that many coding and non-coding RNAs interact with this complex in a size dependent manner.[4]

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-qPCR reaction. Performing triplicate of RT-qPCR reactions per Nuclear RIP sample is also recommended to ensure for the most accurate data.*
    - *If using the relative standard curve method, generate a four point standard curve by making four 5-fold or 10-fold serial dilutions using the RNA from the 10% input sample (section G, step 5) and performing RT-qPCR,. 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 Extension:	60°C 1 min	

J. 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.

Option 1: Normalize DNA concentration to percent of input using relative standard curve

1. For each RNA of interest, perform a serial dilution (10-fold dilution series) 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.

   If using the controls provided in the EZ-Nuclear RIP Native kits (Cat # 17-10523) for the positive control the antibody of interest is the Anti-EZH2 antibody, 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

Option 2. ΔΔCt method

1. Perform quantitative real-time RT-PCR with 2 µL 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 negative control 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 negative control antibody from the ΔCt value of the antibody of interest ($\Delta\Delta Ct = \Delta Ct_{positive} - \Delta Ct_{negative}$).
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-link) 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.

Protocol Summary 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. Lysate Preparation

a. **Prepare Complete Nuclei Isolation Buffer.** For 500 µL of Nucei Isolation Buffer, add 2.5 µL of protease inhibitor cocktail and 1.25 µL of RNase inhibitor. Keep prepared buffer on ice
b. **Prepare Complete RIP Lysis Buffer.** For 100 µL of RIP Lysis Buffer, add 0.5 µL of protease inhibitor cocktail and 0.25 µL of RNase inhibitor. Keep prepared buffer on ice
c. **Harvest cells.** Wash cells twice with ice-cold PBS, then collect cells by centrifugation at 800 x g for 5 minutes at 4°C. Discard the supernatant.
d. **Resuspend the cell pellet** in Complete Nuclei Isolation Buffer. Mix by pipetting up and down several times until the cells have been dispersed and the mixture appears homogeneous.
e. **Incubate on ice for 15 minutes.** Vortex the cell suspension for 10 seconds at high-speed every 5 minutes to enhance cell lysis
f. **(Optional) Homogenize the cell suspension** 10 times in a Dounce homogenizer (loose pestle) to facilitate release of the nuclei
g. **Centrifuge** the cell suspension at 800 x g for 5 minutes at 4°C
h. **Remove supernatant and resuspend the nuclear pellets in Complete RIP Lysis Buffer.** Mix by pipetting up and down several times until the nuclei are dispersed and the mixture appears homogeneous. Incubate on ice for 5 minutes
i. **Pass the nuclei through a 27G needle and freeze at -80°C**

II. Preparation of Magnetic Beads for Immunoprecipitation

a. **Transfer** 10 µL of Magna ChIP protein A/G Magnetic Beads to a microcentrifuge tube
b. **Wash** the magnetic beads twice with 50 µL of Nuclear RIP Wash 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 Wash Buffer
c. **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
d. **Wash** the beads three times with 0.5 mL of Nuclear RIP Wash Buffer. Make sure beads are completely re-suspended in the wash buffer by gentle pipetting between washes
e. **Remove the supernatant** and place the capped tubes on ice

III. DNase I treatment of the nuclear lysate

a. **Thaw the nuclear lysate** (from section I step i) and centrifuge at 10,000 x g for 10 minutes at 4°C. Transfer the supernatant to a new microcentrifuge tube
b. **Add 10 µL of DNase I** to each tube and incubate for 20 minutes at 37°C
c. **Centrifuge** at 10,000 x g for 10 minutes at 4°C and place the tubes on ice

IV. Immunoprecipitation

a. **Prepare Nuclear RIP Immunoprecipitation Buffer.** For 900 µL of Nuclear RIP Immunoprecipitation Buffer, add 5 µL of RNase inhibitor to 895 µL of RIP Wash Buffer
b. **Add 900 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 100 μL of the DNase I-treated nuclear lysate** (from step III. c) to each magnetic bead-antibody complex
d. **Store 10 μL of the DNase I-treated nuclear lysate** (from step III. c) 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 Nuclear RIP 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 5 more times** with 500 μL of ice-cold Nuclear RIP Wash Buffer
k. **Place the tubes on ice and immediately proceed to RNA purification**

V. RNA Purification

a. **Add 1.0 mL of Trizol®** to each microcentrifuge tube from the previous step. Pipette gently to resuspend the magnetic beads
b. **Thaw the input sample (10 μL, step IV. d)** and add 1.0 mL of Trizol®. Mix well
c. **Incubate all the tubes at room temperature for 5 minutes**
d. **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
e. **Transfer the same volume 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
f. **Centrifuge at 12,000 x g for 10 minutes at 4°C** and discard the supernatant carefully
g. **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
h. **Resuspend the pellets** in 16 μL of Nuclease-free water and place the tubes on ice

VI. DNase I Digestion

a. **Prepare the 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 at 37°C for 20 minutes**
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** – refer to section I in detailed protocol for complete description of data analysis methods

Product Performance

Figure 2: Performance of Magna Nuclear RIP 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).

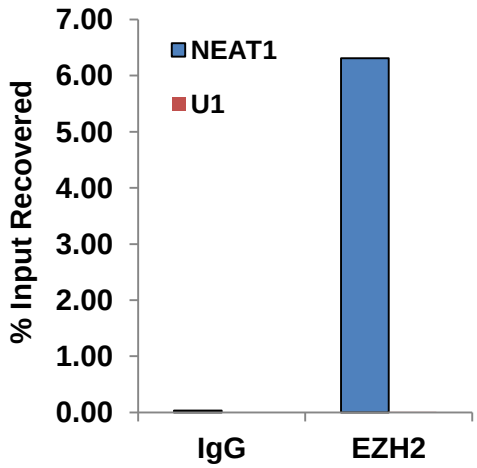


Figure 3 Performance of Magna Nuclear RIP Kit, SUZ12. Nuclear RIP was performed using HeLa cell lysate and either anti-SUZ12 (Part # 03-179) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for NEAT1 (Positive target, Part # CS216139) and U1snRNA (Negative Target, Part. # CS203215).

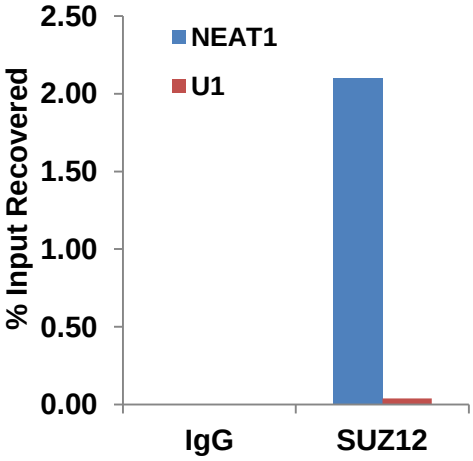


Figure 4: Association of mRNA from Native chromatin to EZH2. Nuclear RIP was performed using HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for GAPDH

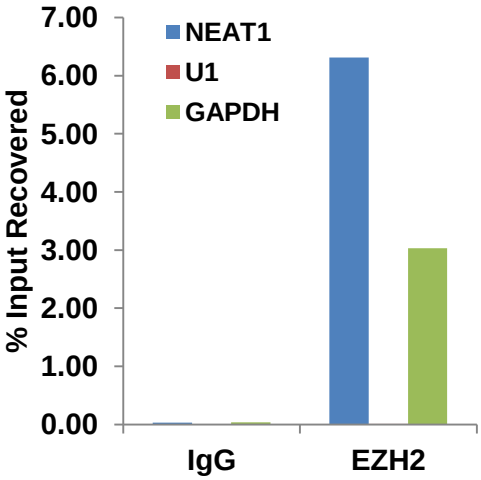
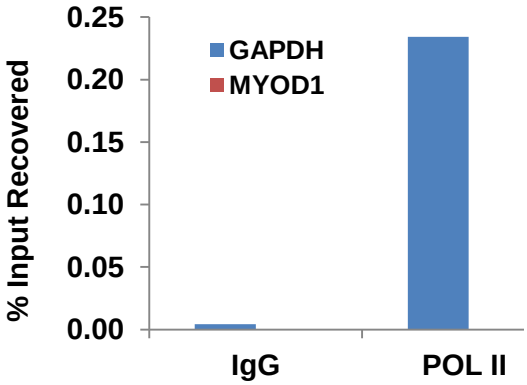
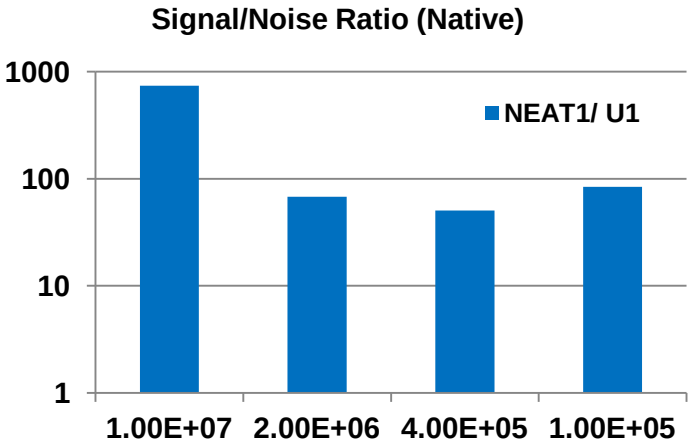


Figure 5: Performance of Magna Nuclear RIP Kit Native using anti-RNA Pol II. Nuclear RIP was performed using HeLa cell lysate and either anti-RNA Polymerase II (Part # 06-523) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was then analyzed by qRT-PCR using RIP Primers specific for GAPDH (Positive target) and MYOD1 (Negative Target).





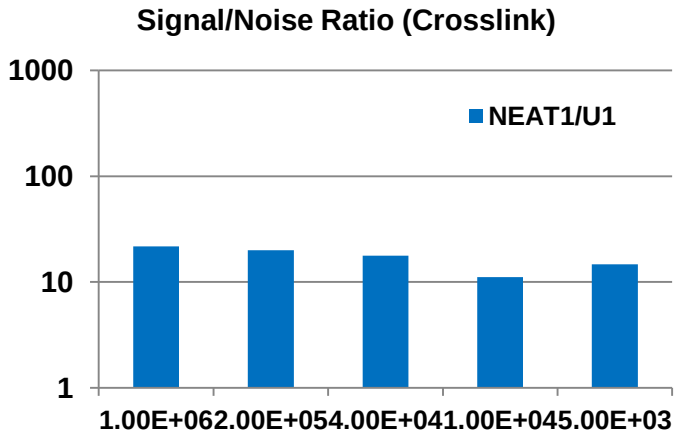
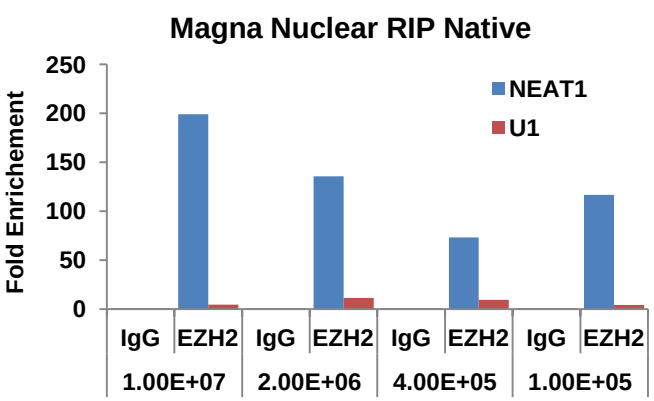


Figure 6: Comparison Native with Cross Linked Methods on Signal to Noise Ratio. Nuclear RIP was performed with (Native) and (Cross-link) kits using various amounts of HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified 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.



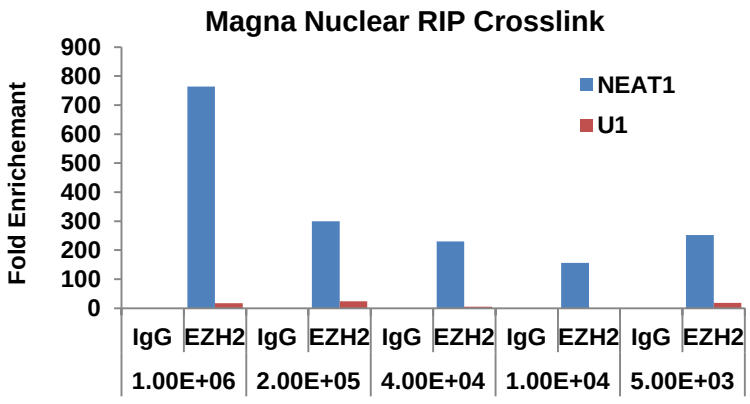


Figure 7: Comparison Native with Crosslink Methods on Sensitivity. Nuclear RIP was performed with (Native) and (Cross-link) kits using various amounts of HeLa cell lysate and either anti-EZH2 (Part # CS203195) or Normal Mouse IgG (Part # CS200621) as the immunoprecipitating antibody. Purified RNA was 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 crosslink protocol NEAT1 enrichment was shown with 5,000 HeLa Cells while with native methods required more cells.

Nuclear RIP Native Optimization and Troubleshooting

Step	Potential Problem	Experimental Suggestions
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 • Titrate antibody and lysate to determine most effective immunoprecipitation conditions by performing IP using 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. Verify that the Magna ChIP Protein A/G magnetic beads are well mixed prior to removing the appropriate volume for IP. • Carefully aspirate liquids when using vacuum aspirator and use a high strength neodymium magnetic rack such as the Millipore Cat. # 20-400 Magna GrIP Rack to ensure Magna ChIP Protein A/G magnetic beads are tightly held against the wall of the microcentrifuge tube.
RNA Purification	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 qRT-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. • Carefully follow the guideline for the RNase control at the beginning of the protocol • Use RNAse inactivating reagents and procedures to ensure work area and materials are RNAse free
	No RNA detected	• Increase incubation time for the isopropanol precipitation. • The RNA ethanol precipitates are sometimes very small and difficult to see. Be sure not to aspirate RNA precipitate when removing the supernatant. Carefully withdraw supernatant from side opposite from location of pellet. • Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis.

Step	Potential Problem	Experimental Suggestions
RT-PCR	No PCR product from Positive Control RIP	• Increase amounts the cDNA added to the PCR reaction up to 10% of the PCR reaction if amount added was less. • Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR with melting curve to confirm amplification conditions and ability of primers to generate a single DNA product peak. • Confirm the antibody can immunoprecipitate the RBP of interest by IP Western prior to RIP analysis
	High background level with negative IP	Insufficient wash after immunoprecipitation. Increase the number of washes of the magnetic beads. Be sure to completely resuspend the magnetic beads between washes. 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.

Related Products

Product	Description	Cat. No.
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
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
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
RIPAb+™ Validated Antibody Primer Set	Proven for RIP or ChIP and lot tested for performance.	Multiple
ChIPAb+™ Validated Antibody Primer Set	See the complete selection at www.millipore.com/antibodies	Multiple

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