Application Note

Effective Nuclear RIP Protocols to Study Interactions between Proteins and Non-Coding RNAs

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, and RNA. Historically, mRNA was considered to be the only RNA associated with chromatin. These mRNAs would transiently associate with chromatin during transcription then exit the nucleus for translation. However, mounting evidence suggests that various classes of non-coding RNAs (e.g. long non-coding RNAs (lncRNA) small nuclear RNAs (snRNA), enhancer RNAs (eRNA) etc.) are associated with chromatin and likely serve regulatory functions[1-3].

For the past several years chromatin immunoprecipitation (ChIP) has been used to interrogate association of proteins with genomic DNA sequences. The need to better understand the RNA component of chromatin has driven the development of additional methods to allow analysis and characterization of chromatin associated RNA. One approach used to detect and identify RNA molecules that interact with a specific protein is RNA-binding protein immunoprecipitation (RIP)[4]. This method allows the immunoprecipitation of protein:RNA complexes that are both nuclear and cytoplasmic using whole cell lysates generated using kit such as the Magna RIP™ RNA Binding Protein Immunoprecipitation Kit.

Chromatin must be obtained from isolated nuclei to eliminate cytoplasmic RNA: protein complexes that can confound analysis. Recognizing that researchers were seeking optimized protocols tailored to the isolation and detection of protein-associated non-coding RNAs in chromatin[5], Merck Millipore has developed the Magna Nuclear RIP™ kits for the isolation, detection, and identification of chromatin-associated ncRNAs and mRNAs.

As a test case for the development of these protocols, lncRNA transcripts associated with the PRC2 complex (Polycomb Repressive Complex 2) were tested for association with PRC2 complex proteins EZH2 and SUZ12. Using both RT-qPCR and RNA-seq, the association of NEAT1 and HOTAIR lncRNAs with PRC2 were examined. The Magna Nuclear RIP™ kits were shown in this study to allow flexibility in the amounts of starting material and type of chromatin needed, provide high signal-to-noise ratios compared to competing assays, and enable the use of either cross-linked or non-cross-linked (native) chromatin. Options for cross-linked or native nuclear RIP enable analysis of high and low affinity interactions as well as direct interactions (e.g. RNA:protein:DNA) or indirect interactions (e.g. RNA:protein:protein:DNA) as often found in multi-subunit protein complexes.

The cross-linked nuclear RIP approach uses chromatin prepared from cells treated with a cross-linking agent (formaldehyde) to preserve protein:DNA, protein:protein, and protein:RNA interactions. The native method involves a simpler protocol with milder lysis and washing conditions designed to preserve stronger RNA-protein

interactions. While the less direct interactions can be preserved by adjusting the stringency of lysis and wash conditions, the cross-linked method is preferred (Figure 1 and Table 1).

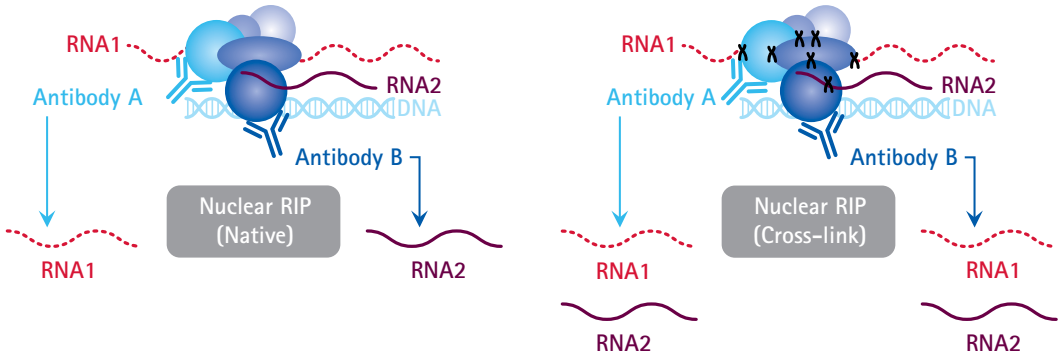


Figure 1.

Mechanistic comparison: mapping protein:RNA interactions using Magna Nuclear RIP™ (native) kit vs. Magna Nuclear RIP™ (cross-link) kit. Chromatin is prepared as the substrate for immunoprecipitation in both methods, although the details of the isolation and fragmentation procedure differ. Native RIP will typically recover high affinity, direct interactions between proteins bearing RNA-binding motifs and candidate RNAs, whereas crosslinking RIP can capture lower affinity protein:RNA interactions within higher molecular weight complexes that likely maintain their in vivo configurations.

Comparison: Magna Nuclear RIP™ (native) kit vs. Magna Nuclear RIP™ (cross-link) kit

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	Yes (preferred method)
Control of lysis and wash stringency to evaluate affinity of interactions	Yes (preferred method)	No
Ability to capture indirect RNA:protein interactions within multiprotein complexes	Possible by controlling wash stringency	Yes (preferred method)
Shearing method	Hydrostatic shearing (freeze/thaw)	Sonication

Table 1.

Comparing assay characteristics and methods between native and cross-linked RIP kits. Based on the differences between the assay mechanisms shown in Figure 1 and the table above, the approach chosen will depend on the characteristics of the interactions to be studied.

Methods and Materials

Antibodies

- ChIPAb+™ EZH2, clone AC22 antibody/primer kit (Cat.No. 17-662)
- RIPAb+™ SUZ12, clone 2A09 antibody/primer kit (Cat. No. 03-179)
- ChIPAb+™ Anti-Pol II antibody/primer kit (Cat. Nos.17-620 and 17-672)

Cross-linked chromatin preparation and RIP

Cells were cross-linked with 0.3% formaldehyde for 10 minutes. Nuclei were extracted and chromatin was prepared with sonication. DNA was digested with DNase I. RIP was performed by incubating chromatin with antibodies listed above and Magna ChIP® protein A/G magnetic beads overnight at 4 °C. Beads were washed with stringent buffers. Cross-links were reversed and RNA was extracted from the samples. After residual DNA was digested with DNase I, samples were analyzed by RT-qPCR. (The method outlined here is provided in the Magna Nuclear RIP™ Cross-Linked Assays User Guide (Cat. Nos. 17-10520 and 17-10521).

Native chromatin preparation and RIP

Cell nuclei were extracted and lysed as directed in the assay instructions. The lysates were passed through a 27 gauge needle four times and digested with DNase I. RIP was performed by incubating chromatin with antibodies listed above and Magna ChIP® protein A/G magnetic beads overnight at 4 °C. Beads were washed with mild wash buffer and RNA was extracted. After residual DNA was digested with DNase I, samples were analyzed by

qRT-PCR. (The method outlined here is provided in the Magna Nuclear RIP™ Native Assays User Guide (Cat. Nos. 17-10522 and 17-10523).

qRT-PCR

Percent input recovery was calculated by standard curve drawn with 10% input sample or delta Ct methods. Specific amplification of the target RNAs was confirmed by melting curve analysis. Ratio of % recovery of positive and negative targets were calculated by simply dividing the % recovery of the positive target by % recovery of the negative target.

Next Generation Sequencing

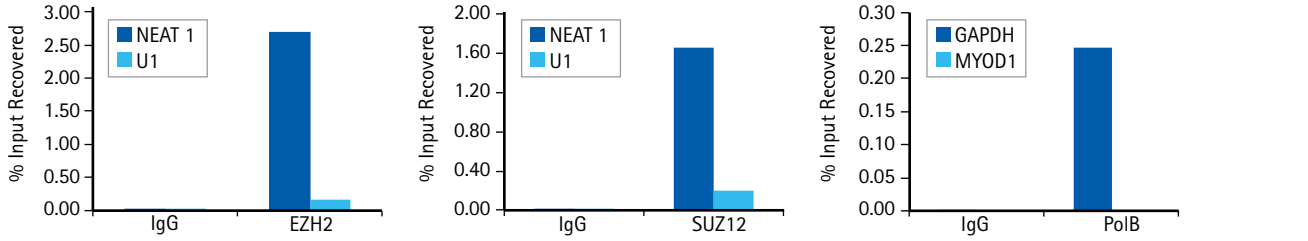
Sequencing libraries were constructed with Encore® Complete RNA-Seq Library System (NuGEN). The resulting libraries were sequenced on the HiSeq instrument (Illumina), aligned and mapped to the human reference genome (hg19). Then RPKM was calculated with Gencode's V17 annotation. The NEAT1 and HOTAIR transcripts from confidently mapped reads browsed with UCSC Genome Browser.

Results

Similar to chromatin immunoprecipitation (ChIP), a high percentage recovery relative to the input chromatin used as well as a high fold enrichment compared to that of a known control signal were two key performance parameters that were optimized during development of the Magna Nuclear RIP™ methods. To help achieve this, protocols and reagents were optimized to reliably provide high signal-to-noise ratios and percent recoveries of chromatin. To achieve this we established positive controls using antibodies against EZH2 and SUZ12. The two proteins detected by these antibodies are known to associate with the lncRNA NEAT1[6,7]. Using these targets as positive controls we optimized buffer conditions and protocols to allow the use of both cross-linked and non-crossed linked chromatin for nuclear RIP.

Examples of data generated using the established protocols is shown in Figure 2. These results demonstrate that the Magna Nuclear RIP™ protocols generate reliable data with strong positive signals and very low backgrounds from either cross-linked or native chromatin samples.

2A. Cross-linked RIP

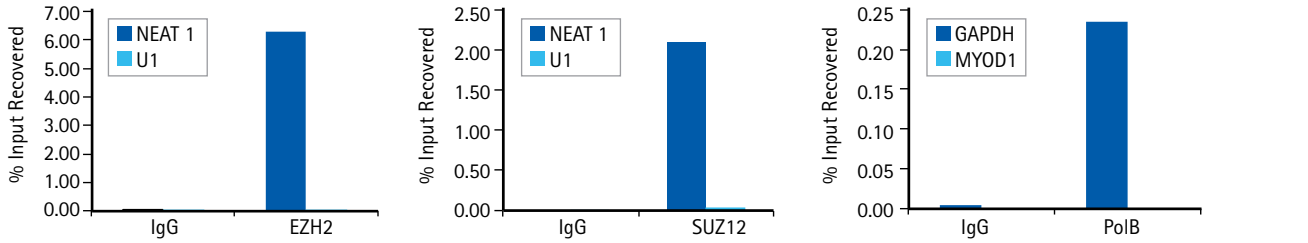


	NEAT1 (%)	U1 (%)	Signal/Noise
IgG	0.02	0.03	NA
EZH2	2.77	0.12	23.1
Fold	138.5	4	346.25

	NEAT1 (%)	U1 (%)	Signal/Noise
IgG	0.003	0.01	NA
SUZ12	1.66	0.2	8.3
Fold	170	4	42.5

	GAPDH (%)	MYOD1	Signal/Noise
IgG	0	0	NA
POL II	0.25	0	ND

2B. Native RIP



	NEAT1 (%)	U1 (%)	Signal/Noise
IgG	0.03	0.002	NA
EZH2	6.31	0.01	631
Fold	210	5	42

	NEAT1 (%)	U1 (%)	Signal/Noise
IgG	0	0	NA
SUZ12	2.1	0.04	52.5

	GAPDH (%)	MYOD1	Signal/Noise
IgG	0	0	NA
POL II	0.23	0	ND

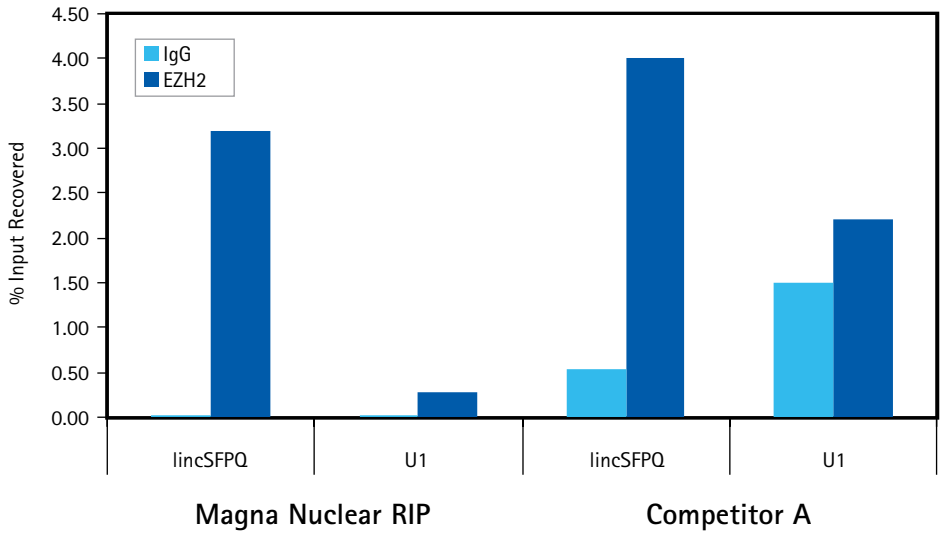
Figure 2.

Specific Enrichment of Chromatin Associated RNA Using Native and Cross-Linked Protocols. Transcripts associated with the PRC2 complex subunits EZH2 and SUZ12 (NEAT1 (positive) and U1snRNA (negative)) and associated with RNA Pol II (GAPDH mRNA (positive) and MYOD1 mRNA (negative)) in HeLa cells were analyzed by qRT-PCR following RIP with Magna Nuclear RIP™ cross-linked (A), and Magna Nuclear RIP™ native (B) kits.

Using both native and cross-linked chromatin, Nuclear RIP using antibodies against PRC2 complex proteins EZH2 and SUZ12 followed by detection of lncRNA NEAT1 (positive signal) by RT-qPCR was performed. The data shown demonstrate high percent recoveries relative to input, as well as low background signals resulting in high fold enrichment values of greater than 130 relative to the IgG background control. Note that in one case (Figure 2B, panel 2, SUZ12 native sample) the IgG background was too low to detect making a fold enrichment calculation impossible. The U1snRNA used as a negative control showed low levels of recovery relative to input and low fold enrichment as expected. Using the U1 snRNA percent recovery data signal-to-noise ratios over 20 were typical in the samples tested (Figures 2A, 2B).

The Magna Nuclear RIP™ kits can not only be used to detect chromatin associated non-coding RNA, but also mRNA associated with chromatin. This is demonstrated in Figure 2 A and B panel 3. For this experiment an antibody against RNA polymerase II was used to recover regions of chromatin actively being transcribed. RT-qPCR using primers against GAPDH mRNA (positive target) and MYOD1 (negative target) were used to confirm the presence of GAPDH mRNA associated with chromatin.

The performance of the Magna Nuclear RIP™ cross-linked kit was compared to that of a chromatin associated RNA immunoprecipitation kit from Supplier A (Figure 3). For these experiments we used an antibody against the PRC2 complex protein EZH2 followed by RT-qPCR. Here U1 snRNA was used as a negative control and lincSFPQ RNA, as shown in supplier A's user manual, was used to demonstrate specific enrichment. In both cases good percent recoveries were obtained. However, the IgG background using supplier A's protocol and reagents was significantly higher than the Magna Nuclear RIP™ kit. Surprisingly, Supplier A's kit showed strong enrichment of the U1 snRNA negative control. As a result of these high background values, the Magna Nuclear RIP™ fold enrichment values were 20 times higher and the signal-to-noise ratios more than 5 times higher than that of Supplier A. Because supplier A does not offer a kit for native nuclear RIP, the Magna Nuclear RIP™ native protocol was not compared.



	Magna Nuclear RIP™		Competitor A	
	lincSFPQ	U1	lincSFPQ	U1
IgG	0.02	3.19	0.54	1.50
EZH2	3.19	0.28	4	2.21
Fold Enrichment	184.9	14	7.42	1.47
lincSFPQ/U1	11.39		1.81	

Figure 3.

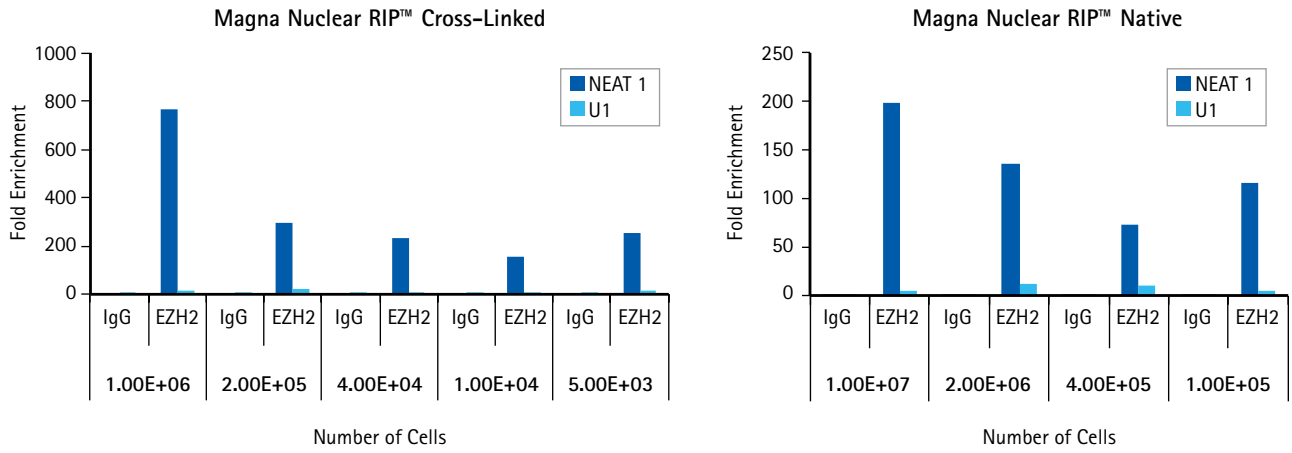
Performance Comparison of the Magna Nuclear RIP™ Kit (Cross-Linked) with Other Commercially Available Kit. Using anti-EZH2 antibody and lncSFPQ as a positive target and U1 snRNA as a negative target, both protocols were performed as per the supplier's instructions. Fold enrichment, signal-to-noise ratios, and specificity of recovery were compared with those obtained using a competitor kit.

The Magna Nuclear RIP™ assays' ability to use either cross-linked or native chromatin substrate for experimental tests provides a new flexibility not previously available. To evaluate the range of input chromatin required for detection experiments using decreasing amounts of input chromatin were conducted (Figure 4). Using the Magna Nuclear RIP™ Cross-linked kit with anti-EZH2 followed by RT-qPCR detection of NEAT1 high fold enrichment and signal to noise ratios were obtained with as few as 5,000 cells or as many as 1,000,000 cells (Figure 4A and B left panels). When using the native kit with anti-EZH2 reliable detection of the NEAT1 RNA was obtained from as few as 100,000 cells or as many as 10,000,000 cells (Figure 4 A and B right panels).

The data in figure 4 demonstrate that both native and cross-linked approaches work over a wide range of cell numbers. However, when cross-linked chromatin was used as a substrate, fewer cells were needed to generate equivalent signals compared to native chromatin. This difference may be a consideration when conducting experiments where cell numbers are limiting.

While both protocols provide robust and consistent signal-to-noise ratios (Figure 4B). With cross-linked substrates, the signal to noise ratios were about 20:1. (Figure 4B, left panel). With native, non-cross-linked substrates, the signal-to-noise ratios were in the range of 50:1 to 700:1 (Figure 4B, right panel). The stronger signal-to-noise ratios seen with native chromatin could be important for experiments where target signal relative to the negative control signal needs to be maximized.

4A.



4B.

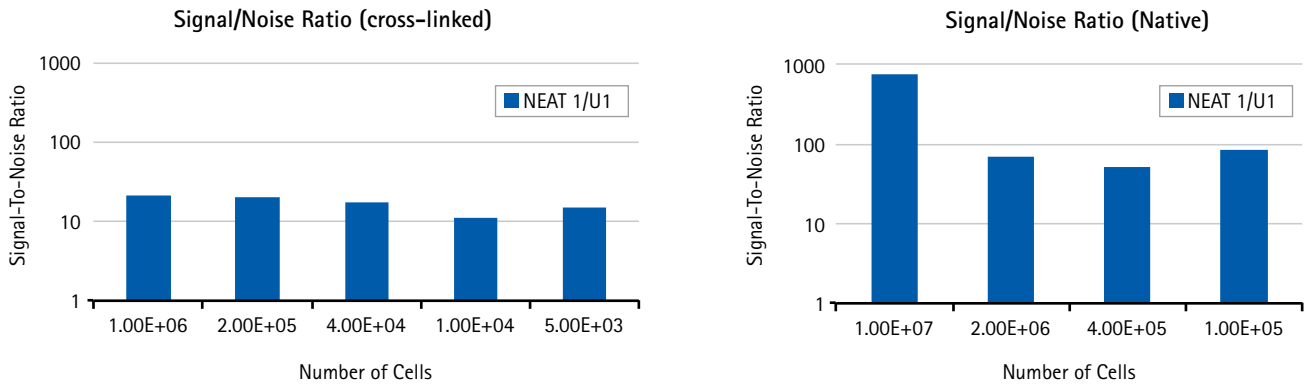


Figure 4.

RIP analysis for NEAT1 was performed using cross-linked (left panels) or native chromatin (right panels) from varying numbers of HeLa cells (A). High and consistent signal-to-noise ratios were achieved with both substrates (B).

To enable genome wide profiling and enable discovery of novel chromatin associations, the Magna Nuclear RIP™ kits were validated using RNA-seq. To verify specific binding EZH2 and SUZ12 proteins were used as targets. These proteins are known to associate with NEAT1 and HOTAIR lncRNAs[6,7]. The results shown in Figure 4 are consistent with published literature[6,7,8] and demonstrate that the Magna Nuclear RIP™ protocols yield RNA samples of sufficient quality and quantity for RIP-seq analysis using either native chromatin or cross-linked chromatin samples.

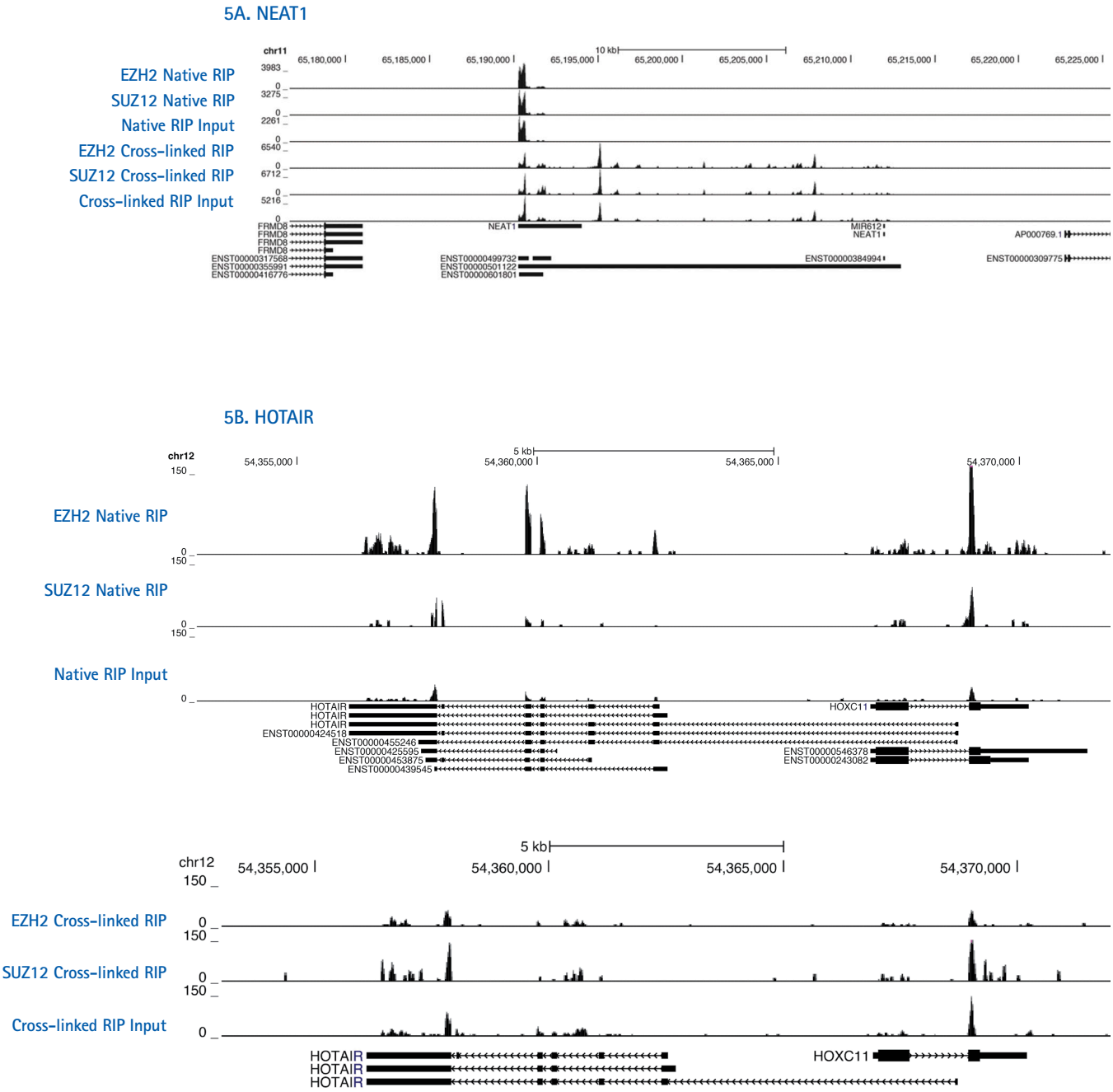


Figure 5.

Using the Magna Nuclear RIP™ cross-linked protocol or the Magna Nuclear RIP™ native protocol and anti-EZH2 clone AC22 (Cat. No. 17-6620) and anti-SUZ12, clone 2A09 (Cat. No. 05-1317), nuclear RIP was performed with HeLa cells. Following isolation, immunoprecipitated RNA was subjected to RNA-seq. Data shown are consistent with binding of NEAT1 (A) and HOTAIR (B) lncRNAs to complexes containing EZH2 and SUZ12.

Discussion

The data presented here show that RIP-based protocols and procedures can be effectively optimized for the isolation and detection of protein-associated long non-coding RNAs (lncRNAs) in chromatin. The Magna Nuclear RIP™ assay protocols and reagents used here demonstrate excellent signal-to-noise ratios through multiple samples that not only varied in original cell count used but also in the type of substrate treatment (cross-linked vs. native). The data show that as few as 5,000 cells could be used with cross-linked kit and as few as 100,000 cells could be used with native kit to generate a robust signal. Either cross-linked or native chromatin substrates yielded high signal-to-noise ratios that were over 20 times higher than those obtained using an assay kit from another commercial supplier.

The flexibility to use either native or cross-linked chromatin is designed to allow researchers to effectively examine high affinity, low affinity, direct and indirect interactions of chromatin-associated RNA with equal confidence in their results.

Data were generated using the same antibodies and primer set using both cross-linked and native chromatin prepared with the respective Magna Nuclear RIP™ kits. The data show that the use of cross-linked chromatin may allow researchers to use fewer cells. Conversely, data generated using native chromatin provided very clean signals but required 10 times as many cells per experiment. Using RIP-Seq to perform a cursory survey of lncRNA-chromatin interactions showed that native chromatin substrate samples yielded different patterns of signal enrichment compared to cross-linked chromatin suggesting that differences in binding affinity could be considered in RIP-seq experiments. The different binding affinity between EZH2 and SUZ12 or between direct interaction (native) and within a complex (cross-linked) is consistent with recent report by Jeannie Lee's laboratory[8]. With these new optimized protocols, researchers may now ask deeper questions involving chromatin and regulatory RNA and potentially be able to better interpret the biological significance of their results.

References

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Ordering Information

Product	Description	Cat. No.
Magna Nuclear RIP™ Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit	Reagents for 24 nuclear RIP reactions using cross-linked chromatin. Includes required chromatin prep, RIP reagents, enzymes, inhibitors and buffers.	17-10520
EZ-Magna Nuclear RIP™ (Cross-Linked) Nuclear RNA-Binding Protein Immunoprecipitation Kit	As above plus positive and negative control antibodies (EZH2, IgG) and amplification primers (NEAT1).	17-10521
Magna Nuclear RIP™ (Native) Nuclear RNA-Binding Protein Immunoprecipitation Kit	Reagents for 24 nuclear RIP reactions using native chromatin. Includes required chromatin prep, RIP reagents, enzymes, inhibitors and buffers.	17-10522
EZ-Magna Nuclear RIP™ (Native) Nuclear RNA-Binding Protein Immunoprecipitation Kit	As above plus positive and negative control antibodies (EZH2, IgG) and amplification primers (NEAT1).	17-10523

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