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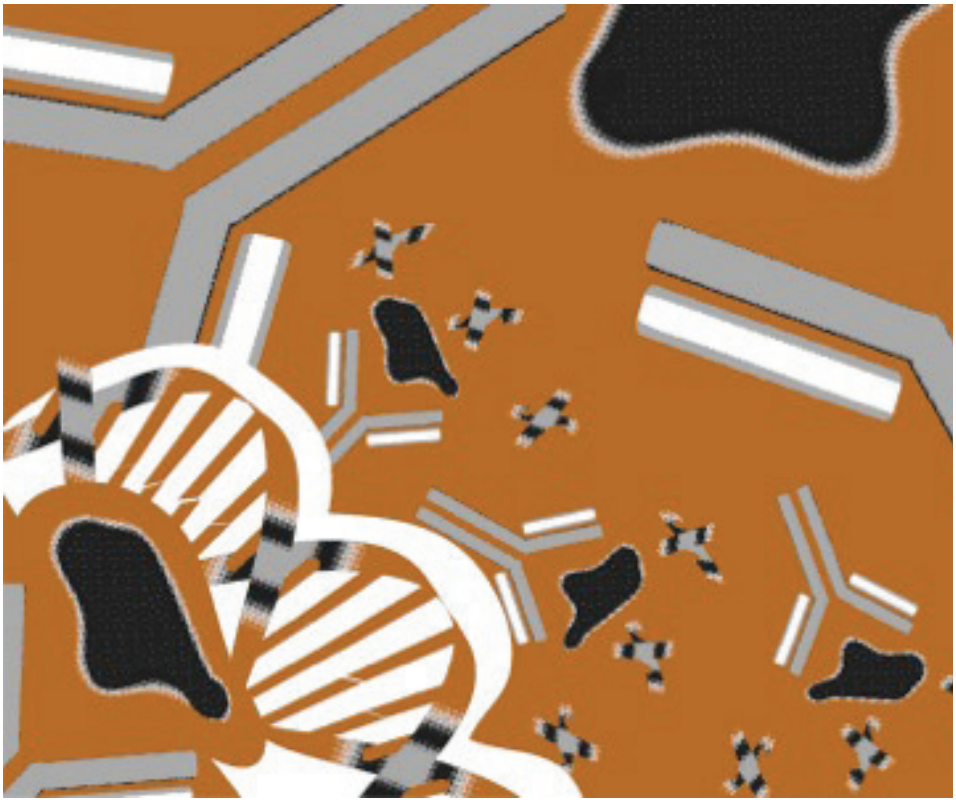
Informally Informative

EpiGenie Guide: RNA Immunoprecipitation (RIP) And Beyond



In this guide…

RNA in a Bind

RNA binding proteins (RBPs) play important roles in many cellular and disease processes, together with their DNA binding brethren, they compromise almost one quarter of annotated human genes. RBPs can act in a highly sequence-specific context and are involved in RNA splicing, editing, translational regulation and RNA degradation. The tools used to understand when and where these proteins bind and interact with RNAs in the cell have lagged behind those for protein-DNA interactions, although they are rapidly gaining ground. This guide will describe some of the more recently used tools to examine protein-RNA binding including the variants of RIP and CLIP and their combination with next-generation sequencing (NGS).

RNA-immunoprecipitation

RNA Binding Protein Immuno-precipitation allows users to analyze protein-RNA binding in the same way as the more familiar Chromatin-immunoprecipitation (ChIP) is used to study protein-DNA binding. Immunoprecipitation with an antibody against a protein of interest enriches for RNA molecules actively bound to the target protein. "The value in RIP is in being able to isolate lysates that are relatively ribosomal RNA free for the purposes of profiling protein-RNA interactions. This method is useful for both cytoplasmic and nuclear protein-RNA interactions from primary or cultured cells," shared Dr. John Rosenfeld, R&D Group Leader at EMD Millipore, a producer of optimized RIP kits and reagents.

Initially RIP studies used a native immunoprecipitation without any form of cross-linking. The conditions required had three significant issues:

- RNAs bound to protein other than the target can potentially co-precipitated
- Reaction conditions preserved only a subset of cellular interactions
- *In vitro* artifactual interactions may be detected formed during the process

To improve the stability of the interactions, cells or tissue can be treated with formaldehyde that generates protein-protein, protein-DNA, and protein-RNA cross-links.

This material is then usually sonicated to produce fragments of 300-500bp and DNAse treatment removes DNA before immunoprecipitation with an antibody against the protein of interest enriches the bound RNA molecules. Early RIP studies analyzed only a few loci; the RNA fragments were used in reverse-transction PCR to identify differential binding of RNAs to proteins one locus at a time, with gel mobility shift assays or using clone library screening for a more comprehensive analysis, but the downstream analysis approach quickly ramped up.

Stepping Up Coverage: RIP-ChIP and RIP-Seq

Although the early RNA immunoprecipitation studies led to many interesting discovers, researchers needed higher coverage analysis gain a richer understanding of RNA regulatory networks. This led researchers to develop assays that allowed the interrogation of bound RNA using microarrays (RIP-ChIP) and subsequently RNA sequencing (RIP-Seq).

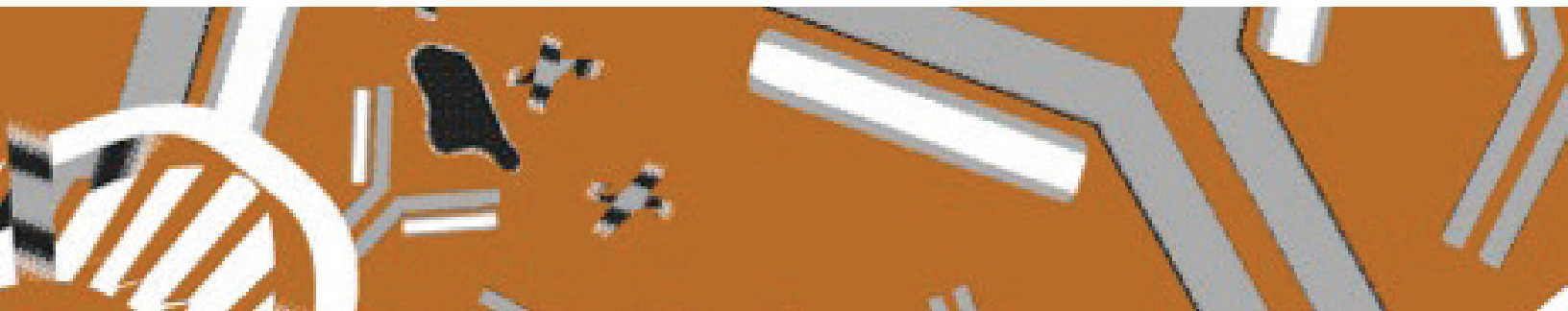
Similar to the transition seen with ChIP, these powerful downstream analysis techniques uncovered diverse and extensive protein-RNA interactions. Daniel Hogan and colleagues provided a glimpse into the RNA interactome in yeast back in 2008 when they found a fraction of the predicted RNA binding proteins bound anywhere from a few to hundreds of target mRNA.

While high-density arrays and high throughput sequencing unleashed the coverage constraints, it became clear in these early studies that the formaldehyde cross-linking had a few hiccups in the approach that could lead to challenges downstream. Robert Darnell detailed some of his team's experiences with RIP and issues with signal to noise ratios in their attempts to investigate the RNA targets of the fragile-X mental retardation protein, FMRP. They found that the approach often lead to co-purification of non-target molecule RNAs. But if formaldehyde cross-linking wasn't used, then RNA and RBPs could easily dissociate and re-associate *in vitro*.

Both of these issues required researchers to use carefully controlled and designed experiments to make the most of RIP. Fortunately, advances in cross-linking were on the horizon.

Getting into the Specifics with CLIP: Cross-Linking Immunoprecipitation

UV cross-linking and immunoprecipitation (CLIP) was developed by Jernej Ule at Howard Hughes Medical Institute while in Robert Darnell's group. CLIP makes use of the photoreactivity of pyrimidines and specific amino acids to produce irreversible covalent crosslinks between RBP's and their bound RNA's, while completely avoiding protein:protein cross-linking.



CLIP can be performed on live cells preserving a snapshot of a specific in vivo environment and is done rapidly at 4C using UV irradiation. It has been suggested that the rapidity of treatment allows very little biological reaction to occur, which can confound experimental results, however it is worth considering the impact of DNA damage responses if the process is completed on cells that will be kept living after treatment.

With CLIP, it's possible to use a rigorous purification, which can be helpful in removing artifacts. The fragments of RNA recovered after immunoprecipitation can then be cloned, amplified by PCR and, in the original paper were Sanger-sequenced.

But some experts suggest to proceed with caution.

"Like the chromatin cross-linked based methods, there can be higher background when dealing with these samples, and acrylamide isolation of enriched protein-RNA is a key step in this process to eliminate both protein partners in a complex, as well as extraneous RNA products."

Dr. John Rosenfeld
R&D Group Leader, EMD Millipore

CLIP-Seq

There haven't been many applications that high throughput sequencing couldn't improve. CLIP has been no exception. Next-generation sequencing of CLIP was first demonstrated as high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) by Robert Darnell's group in 2008 using the Roche-454 sequencing platform.

Their analysis of the Nova splicing factor showed how reproducible the technique was. They created genome-wide maps of Nova:RNA binding, and demonstrated that Nova plays a role in alternative polyadenylation in the brain.

The term CLIP-seq was first used by Jeremey Sanford and colleagues from UCSC in 2009. They used CLIP-seq to determine the RNA binding specificity of SFRS1, a regulator of pre-mRNA splicing. They showed that many different RNA molecules were bound by SFRS1, not just mRNA's but also micro-, non-coding- and other RNA species; all of which shared a consensus-binding motif. This binding motif was used to screen the Human Gene Mutation Database for mutations disrupting SFRS1 binding where they found nearly 200 mutations in over 80 genes suggesting a role for aberrant splicing in Human disease.

But, like many methods CLIP isn't infallible. Markus Hafner and colleagues review some of the issues with CLIP. The wavelength of crosslinking in CLIP (UV 254 nm) isn't always efficient and the mapping the precise location of the cross-linked material isn't easy. This can make separating the relevant interactions from some of the carryover RNA difficult.

On PAR-CLIP: Photoactivable Ribonucleotid Enhanced CLIP

To address some of the issues experienced with the first generation CLIP protocol, Hafner and colleagues developed, PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation), which modified CLIP further by incorporating photoreactive nucleotide analogs, 4-thiouridine (4-SU) and 6-thioguanosine (6-SG), into RNA molecules during transcription. UV irradiation at 365nm was then used to induce a very efficient crosslinking of the now photoreactive cellular RNAs and the proteins to which they were bound. A nice upside to the PAR-CLIP approach is that it's possible to precisely map the position of crosslinking, which helps resolve some of the analysis sometimes seen with CLIP. This benefit comes from the fact that crosslinking the 4-SU analog results in thymidine to cytidine transitions, where as crosslinking the 6-SG analog results in guanosine to adenosine mutations. These mutations make it easier to separate the crosslinked RNA from other, more abundant cellular RNA. The downside? PAR-CLIP is currently limited to *in vitro* cell culture studies and the analogs used are not perfectly tolerated by cells.

iCLIP: Individual Nucleotide Resolution CLIP

No, Apple hasn't sprung into the RNA analysis market. . .yet. iCLIP (individual-nucleotide resolution CLIP), is another interesting innovation that improves the efficiency of CLIP by replacing an inefficient RNA ligation step with a more efficient cDNA circularization and at the same time adds a random barcode to the DNA adaptor. Sometimes cDNA synthesis from CLIP libraries can truncate at the crosslinked loci. This can be a problem, but iCLIP uses it to its advantage; by capturing these truncated cDNAs, it is possible to map the cross-linked contacts with single nucleotide precision. iCLIP is a useful CLIP variant, but like other approaches, it comes with some fine print. There are a number of enzymatic and purification steps to work through, which means there are plenty of opportunities for things to go wrong, so you'll want to use a host of QC/checkpoints. The authors make some good suggestions in their discussion that should serve as a useful guide.

Summary of the CLIPings

Although the early CLIP-seq studies allowed the first comprehensive genome-wide maps of protein-RNA interaction to be generated, they weren't flawless. Several deficiencies in these methods were observed by the authors including the need for significantly higher sequencing depth and the impact of PCR artifacts on quantitation of individual transcripts. That said, the majority of CLIP-seq projects have been run on Illumina sequencing instruments that today can generate 3B reads per run, allowing 100's of samples to be analyzed in just a few days. We expect continued innovation in this exciting area.

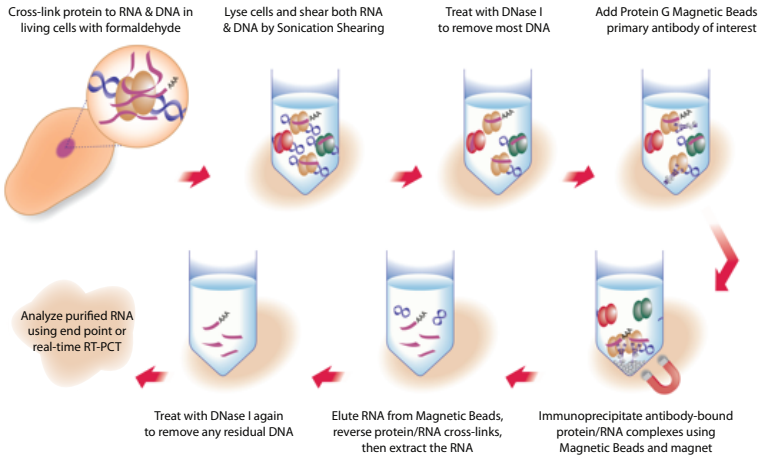
Stepping into Chromatin: ChIP

As we've outlined here, there are number of ways to pin down protein-RNA interactions, but sometimes it pays to take the reverse approach and head right to the context. . . like chromatin for instance.

So how does ChIP work when it's RNA you're after? Surprisingly, not that much different as Kelly explains, " When performing ChIP to examine RNA, standard ChIP protocols can be used with minor adjustments such as using RNAse free reagents, modified binding and wash buffers, the inclusion of two DNAse I treatment steps in the presence of Mg2+ and Ca2+ (one before the IP step and one after the purification) to decrease background. Following elution RNA is purified using Trizol, reverse transcribed and analyzed by PCR; the inclusion of a no RT control will measure DNA contamination."

"Several techniques are available to identify protein-RNA interactions, however a direct role for RNA in chromatin regulation through microRNAs and lncRNAs, is becoming increasingly more apparent. Traditionally, chromatin immunoprecipitation is preformed using antibodies to histones or DNA binding proteins followed by identification of the associated DNA regions, however it can be adapted to examine RNAs that are associated with chromatin. The use of fixed material to examine chromatin-RNA interactions stabilizes the dynamic chromatin environment which can increase sensitivity over methods using unfixed material."

Dr. Terry Kelly, Active Motif R&D Manager.



Flow chart of the RNA ChIP-IT™ process.
Live cells are fixed using formaldehyde, which cross-links and preserves protein/RNA interactions (as well as protein/DNA interactions). The RNA/DNA is then sheared into small, uniform fragments using sonication and, after a DNase treatment to remove residual DNA, specific protein/RNA complexes are immunoprecipitated using an antibody directed against the RNA-binding protein of interest. Following immunoprecipitation, cross-linking is reversed, the RNA is extracted then DNase I treated again (to remove residual DNA). The RNA is then analyzed by end point or real-time RT-PCR to determine which RNA fragments were bound by the protein of interest.

John Rosenfeld adds, "There are emerging methods that involve isolating nuclei for the specific purpose of isolating non-coding protein-RNA interactions from chromatin. Nuclear RIP can achieve this without the use of crosslinking agents such as formaldehyde, which can be useful for confirming results obtained from formaldehyde crosslinking experiments."

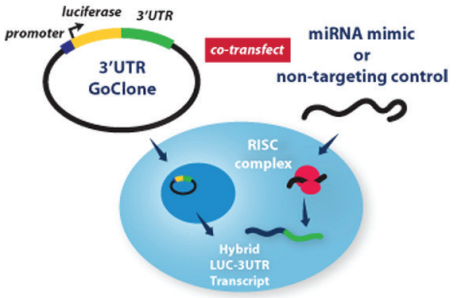
"The sample type for these protocols can either be a native, nuclear RIP lysate where DNA components are degraded, or can be prepared chromatin, which is more like a ChIP reaction in the presence of RNA inhibitors and with DNAse treatment to remove contaminating DNA from chromatin. These methods are complementary, as crosslinking chromatin can elucidate interactions, which may not be high affinity or bona fide binding events. Generally, RIP, Nuclear RIP and Cross-linked IP can utilize qRT-PCR as a readout when a target species is known, or can be applied to library construction for ncRNA-seq discovery experiments," stated Rosenfeld.

Validating RIP Results and Measuring Functional Consequences

"When a protein-RNA interaction is detected in a RIP experiment, it is often desirable to validate the interaction and characterize the functional consequences of that interaction. Since many RNA-binding proteins including the miRNA-RISC complex interact with the 3'UTRs of protein coding transcripts, 3'UTR reporter constructs are a popular way to assess the function of an RNA:protein interaction," shared Dr. Nathan Trinklein, SwitchGear Genomics (an Active Motif company).

In this method a 3'UTR is cloned downstream of a reporter gene on a plasmid vector. When this vector is transfected into a cell, it creates a hybrid transcript that contains the reporter gene fused to the 3'UTR of interest. The 3'UTR reporter construct can be co-transfected with either a cDNA expression vector or a siRNA to a RNA-binding protein of interest to measure the activity of the 3'UTR in the absence or presence of the RNA-binding protein.

Likewise, the 3'UTR reporter vector can be co-transfected with a miRNA mimic or inhibitor to measure the activity of the 3'UTR in the presence or absence of a particular miRNA (see figure below). As a final confirmation, the 3'UTR sequence can be mutated in the reporter vector to either remove a miRNA seed sequence or disrupt a secondary structure necessary for protein binding.



More Tips and Protocol Guidance

For more grit on these methods and other useful tools check out these great reviews and publications:

HITS-CLIP: panoramic views of protein-RNA regulation in living cells
This is an amazing review that covers, CLIP and CLIP-seq and provides tons of insightful commentary on the methods, their utility, and future areas for improvement.

PAR-CLIP - A Method to Identify Transcriptome-wide the Binding Sites of RNA Binding Proteins
This nice pub goes into good depth about some of the issues you may run into with standard CLIP-seq, and introduces the ehancements and utility of PAR-CLIP.

iCLIP - Transcriptome-wide Mapping of Protein-RNA Interactions with Individual Nucleotide Resolution
Here's a nice introduction to iCLIP complete with a some visual razzle dazzle courtesy of Jove.

RIPSeeker: a statistical package for identifying protein-associated transcripts from RIP-seq experiments
This nice paper introduces one of the only software packages out there for analyzing RIP-seq data.

Product	Catalog Number
EMD Millipore	
RNA-Binding Protein Immunoprecipitation Kit	
Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	17-700
EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	17-701
Magna RIP™ Quad RNA-Binding Protein Immunoprecipitation Kit	17-704
Active Motif	
RNA ChIP Kits	
RNA ChIP-IT®	53024
RNA ChIP-IT® Control Kit - Human	53025
Reporter Systems for Protein-RNA Interactions	
LightSwitch™ Promoter and 3´UTR Reporter Collections	Variable
LightSwitch™ Luciferase Assay Kit	32031
LightSwitch™ Luciferase Assay Kit	32032
Empty LightSwitch™ Reporter Vectors	Variable