

RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts

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RNA targets of multitargeted RNA-binding proteins (RBPs) can be studied by various methods including mobility shift assays, iterative *in vitro* selection techniques and computational approaches. These techniques, however, cannot be used to identify the cellular context within which mRNAs associate, nor can they be used to elucidate the dynamic composition of RNAs in ribonucleoprotein (RNP) complexes in response to physiological stimuli. But by combining biochemical and genomics procedures to isolate and identify RNAs associated with RNA-binding proteins, information regarding RNA–protein and RNA–RNA interactions can be examined more directly within a cellular context. Several protocols — including the yeast three-hybrid system and immunoprecipitations that use physical or chemical cross-linking — have been developed to address this issue. Cross-linking procedures in general, however, are limited by inefficiency and sequence biases. The approach outlined here, termed RNP immunoprecipitation–microarray (RIP-Chip), allows the identification of discrete subsets of RNAs associated with multi-targeted RNA-binding proteins and provides information regarding changes in the intracellular composition of mRNPs in response to physical, chemical or developmental inducements of living systems. Thus, RIP-Chip can be used to identify subsets of RNAs that have related functions and are potentially co-regulated, as well as proteins that are associated with them in RNP complexes. Using RIP-Chip, the identification and/or quantification of RNAs in RNP complexes can be accomplished within a few hours or days depending on the RNA detection method used.

INTRODUCTION

Global gene expression analysis has received much attention in recent years owing to the growing availability of microarray and other high-throughput sequencing technologies. Most studies have focused on profiling the steady-state levels of mRNA of various cellular, developmental and disease responses using transcriptomics. It is believed, however, that the steady-state levels of mRNAs in a cell do not always directly correlate with the steady-state protein levels^{1,2}. Much of this discrepancy may be accounted for by RNA processing and translational events that occur post-transcriptionally.

Post-transcriptional regulation of gene expression is a ribonucleoprotein-driven process, which involves RBPs and noncoding RNAs (e.g., microRNAs) that affect splicing, nuclear export, subcellular localization, mRNA decay and translation^{3–7}. One of the challenges for understanding post-transcriptional control of gene expression is identifying the RNAs associated with RBPs in a cellular context. Traditionally, mRNA targets have been identified individually using *in vitro* techniques such as cross-linking with ultraviolet light, nitrocellulose filter binding and RNA electromobility shift assays (REMSAs)⁸. Although these methods have provided abundant biochemical information, they are limited in their ability to identify *de novo* targets when starting with an RBP and its unknown RNA targets. Additionally, bioinformatic algorithms have been developed to search for previously uncharacterized mRNA targets of particular RBPs, but such approaches are plagued by the fact that singular RNA binding sites tend to be very small (4–12 nucleotides) and therefore appear more frequently among mRNAs than expected. Biochemical methods for identifying *de novo* targets *en masse* were initially addressed by applying iterative *in vitro* selection against genomic RNA libraries⁹. This led to the elucidation of

over 100 mRNAs derived from human brain cells that were able to bind to the HuB (Hel-N1) RNA-binding protein. But because this was an *in vitro* binding procedure, there was no certainty that the RNAs were ever in the same RNP context or associated directly with the RBP. Thus, although these *in vitro* technologies can identify RNA components and may suggest global binding sites for RBPs, they cannot be used to determine RNA species within the context of cell extracts or the dynamics of RNP interactions after physiological perturbations. One solution to finding *in vivo* RNA substrates of RBPs has been the yeast three-hybrid system^{10–12}. Although this creative adaptation of the yeast two-hybrid system has been used to identify some cognate targets of RBPs, it is limited in that it requires an artificial target reporter, and it has not been used to simultaneously identify multiple targets *en masse*. Moreover, there are limitations to the procedure, which concern the sequence preferences for transcription by yeast RNA polymerase and structural constraints on potential RNA-binding elements. For example, RNAs that are uridylylate-rich tend to cause premature termination by the yeast transcription apparatus, making them unavailable to interact with RBPs that prefer single-stranded U-rich RNA binding sites. Also, secondary structures in RNAs can significantly influence the outcome of a three-hybrid screen. Indeed, important examples of this type of mRNA target discovery have emerged using this procedure¹².

The RIP-Chip (sometimes called RIP-on-Chip or RIP-SEQ) protocol described here allows the identification of multiple RNA targets of RBPs globally and within the context of a cell extract that may or may not have been subjected to physical or chemical cross-linking. To accomplish these goals with cytoplasmic RNAs, we use a mild lysis buffer and optimized conditions, which leave nuclei essentially



intact and minimize inappropriate exchange of mRNAs during subsequent immunoprecipitation^{13–16}. Other conditions that use nuclear extracts have also been adapted for this procedure¹⁷. For standard RIP-Chip, extracts are immunoprecipitated and the pellets washed extensively, the RNP is then released and dissociated into RNA and protein as the RNA is extracted. Once the RNA is purified, it can be detected by various methods including microarray analysis or high-throughput sequencing. More recently, Mili and Steitz demonstrated the ability of *c-fos* mRNA, a known target of HuR, to migrate to HuR within an extract after incubation of extracts from two different cell types¹⁸. Indeed, it has been known for many years that exogenous RNAs can bind to RBPs when added to cell extracts. Thus it is likely that small pools of free RNAs and RBPs are present in all cells, and are available for post-lysis interactions. Our RIP-Chip conditions are optimized to minimize inappropriate interactions^{13–15,19}.

During the development of RIP-Chip we experimented with chemical cross-linking using reversible formaldehyde (potentially a hazardous compound), and found the procedures cumbersome and yielding high background binding²⁰. After the advent of RIP-Chip several related protocols were reported, which use RNA–protein cross-linking to identify RNAs bound to RBPs^{17,21–26}. But we have not found cross-linking to be necessary to identify RNAs associated with RBPs using cell extracts, and prefer to avoid any potential artifacts that may be introduced because these reagents have the potential to severely reduce cell lysis efficiency, to introduce sequence biases, increase background and to be incompletely reversible^{19,24}. More recently, Kaneko and Manley found that using 0.1% formaldehyde rather than 1% formaldehyde for cross-linking RNA to RNA polymerase II significantly improves the quality and recovery of bound RNA¹⁷.

Several factors need to be considered when optimizing a RIP-Chip protocol. First, extracts are kept cold and immediately frozen after lysis and centrifugation. Upon thawing of cell lysates, the extract is diluted tenfold. Next it should be noted that the immunoprecipitation and washing conditions may vary depending on the specific mRNP being investigated. It may be necessary to use more stringent wash conditions such as adding from 0.5 M to 3 M urea, 0.1% or less SDS, or deoxycholate to the wash buffer to reduce background. An additional consideration is the accessibility of the epitope (or tag) to which the immunoprecipitating antibody is directed. Some RBPs may be obstructed to some degree by other components of the RNP complex or to be shielded in a cellular subcompartment. We have found it useful in some instances to use an epitope-tagged RBP rather than endogenous proteins for mRNP isolation. Whereas exogenously expressing a tagged RBP has the potential to affect function, exceedingly high levels of expression are not necessary. For example, the PAB RBP is autoregulated by adenylates in the 5' UTR of its mRNA, and this can balance exogenous and endogenous levels of the protein¹⁹. It may also be possible to further reduce background in a RIP-Chip assay by the use of tandem affinity purified (TAP) tags^{27,28}. Biotinylated tags are also useful in reducing background because they allow for harsher washing conditions because of their high affinity for the streptavidin ligand²⁹. Furthermore, as discussed below, the introduction of EDTA to the immunoprecipitation reaction can be useful for reducing background and gaining access to epitopes, presumably by dissociating ribosomes from mRNP complexes^{13–16,30}.

Once RNA is extracted from the isolated mRNP complexes, there are several methods that may be used to identify the global set of

mRNA targets. As far as we have been able to assess using RT-PCR, the mRNAs recovered using RIP-Chip are intact and of full length. Originally, spotted arrays with a few hundred genes were used to develop these procedures¹³. But alternative detection technologies as well as newer generations of arrays have been introduced that have also been applied to RIP-Chip. For example, a new technology that may be used to detect mRNA targets involves massively parallel sequencing such as single molecule platform sequencing (SMPS)³¹. These high-throughput sequencing methods are currently more expensive, but they offer a more complete and unbiased assessment of the global population of RNAs associated with a RNP complex. Another more recent technology is 'tiling arrays', which consist of oligonucleotides spanning the sequences of entire chromosomes³². Tiling arrays, when combined with RIP-Chip, can detect coding as well as small noncoding RNA species present within RNP complexes. Moreover, tiling arrays can allow the detection of discrete splice variants that associate with RBPs, and in combination with partial digestion or fragmentation of the mRNA, may allow precise mapping of RBP binding sites within the RNAs to which they are directly bound (S.A. Tenenbaum, P. Kapranov, T.R. Gingeras and J.D.K., unpublished observations). Additionally, RIP-Chip, together with microarrays designed to detect microRNAs, can be used to identify the microRNAs that copurify with RNP complexes (P.J. Lager, J.M. Thomson, U. Ohler, S.M. Hammond and J.D.K., unpublished data)³³.

The protocols outlined here concern the preparation of cell extracts and the recovery of messenger ribonucleoprotein (mRNP) complexes to identify subsets of mRNAs and other noncoding RNAs *en masse* that are potentially coregulated. To prepare high-quality materials that contain intact pre-mRNPs or mature mRNPs, good biochemical practices are essential. This includes keeping materials on ice and adding inhibitors of ribonucleases (RNases) and proteases at all steps except where noted. For this protocol, preparing the mRNA lysate and coating protein A/G beads with antibody may be performed in advance of the immunoprecipitation reaction and RNA precipitation, or all steps can be performed sequentially as a single procedure.

The RIP-Chip protocol was also developed to identify RNA components associated with mRNP complexes under changing cellular conditions while minimizing some of the concerns such as sequence bias and high backgrounds known to be a problem with other protocols mentioned above. When designing a RIP-Chip experiment there are several issues to be considered for customizing optimal performance. First, prior knowledge of the biochemical properties of the mRNP complex, including specificity for individual targets and behavior of the complex, can be valuable in optimizing the protocol. For example, 'is the RBP of interest abundant?', 'does it have known sequence preferences?' and 'in which cellular compartment is it localized?' It can be useful to have prior knowledge of the approximate affinity of the RBP for its RNA substrates, although affinities *in vivo* are rarely known. To date, RIP-Chip has proved quite robust with dozens of RBPs, but the protocol may not be optimal for detecting weak associations or indirect interactions, as little prior knowledge is available in this regard. Another potential concern with all protocols like RIP-Chip is that the endpoint detection method can be expensive, and it may therefore be astute to incorporate a series of pilot experiments using single RNA detection methods (e.g., RT-PCR) while testing multiple control RNAs before investing in a genome-wide experiment^{13,14}.

MATERIALS

REAGENTS

- 1 M dithiothreitol (DTT; Fermentas, cat. no. RO861)
- Ethylenediamine tetraacetic acid, EDTA (EM Science, cat. no. 4005)
- Glycogen (Roche, cat. no. 10901393001)
- 1 M HEPES (pH 7.0; Sigma, cat. no. H3375)
- Igepal Nonidet P-40 (NP40; Sigma, cat. no. I-30201)
- 1 M KCl (Mallinckrodt, cat. no. 8648)
- 1 M MgCl₂ (EMD Bioscience, cat. no. MX0045-1)
- NT2 buffer (see REAGENT SETUP)
- Polysome lysis buffer (see REAGENT SETUP)
- Protease inhibitor cocktail tablets (Roche, cat. no. 11697498001)
- Protein A, immobilized on Sepharose (Sigma, cat. no. P3391)
- Proteinase K (Roche, cat. no. 1964364)
- RNase Out RNase inhibitor, 100 units/ml (Invitrogen, cat. no. 10777-019)
- 1 M NaCl (Mallinckrodt, cat. no. 7581)
- Sodium dodecyl sulfate (SDS; EMD Bioscience, cat. no. 7910)
- 1 M Tris-HCl (pH 7.4; JT Baker, cat. no. 4103-01)
- Trizol (Invitrogen, cat. no. 15596-026)
- Urea (Mallinckrodt, cat. no. 8648)
- Vanadyl ribonucleoside complexes (VRC; New England Biolabs, cat. no. S1402S)

REAGENT SETUP

Solutions Prepare all of the above solutions and buffers in RNase-DNase-free H₂O.

Polysome lysis buffer

- 100 mM KCl
- 5 mM MgCl₂
- 10 mM HEPES (pH 7.0)
- 0.5% NP40
- 1 mM DTT
- 100 units ml⁻¹ RNase Out
- 400 μM VRC

Protease inhibitor cocktail

To make 5 ml of polysome lysis buffer, add 50 μl of 1 M HEPES (pH 7.0), 500 μl of 1 M KCl, 25 μl of 1 M MgCl₂ and 25 μl of NP40 to 4.7 ml of RNase-DNase-free H₂O. Add 50 μl of 1 M DTT, 12.5 μl of 100 U/ml RNase Out, 200 μl of Protease inhibitor cocktail (dissolve tablets according to the manufacturer's instructions) and 10 μl of 200 mM VRCs at the time of use.

NT2 buffer

- 50 mM Tris-HCl (pH 7.4)
- 150 mM NaCl
- 1 mM MgCl₂
- 0.05% NP40

To make 50 ml of NT2 buffer, add 2.5 ml of 1 M Tris (pH 7.4), 7.5 ml of 1 M NaCl, 50 μl of 1 M MgCl₂ and 25 μl NP40 to 40 ml of RNase-DNase-free H₂O.

PROCEDURE

Preparation of mRNP lysate ● TIMING ~1 h

1| Collect enough tissue culture cells to generate 2–5 mg of total protein per RIP. Typically, this is comparable to 5–20 × 10⁶ mammalian cells. Pellet by centrifugation (~1,000g) for 10 min at 4 °C, washing several times with 10 ml of ice cold phosphate-buffered saline (PBS) in a conical tube. Alternatively, whole tissue may be ground using a mechanical homogenizer. Additionally, individual cells derived by micro-dissection may be used. The 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.

2| Resuspend final cell pellet with an approximately equal volume of polysome lysis buffer supplemented with RNase inhibitors and protease inhibitors (see REAGENT SETUP). Clumps of cells should be broken up by pipetting up and down several times. Allow mRNP lysate to incubate on ice for 5 min and store at -100 °C. Lysate may be stored for several months at -100 °C. The lysis of certain cell types can be enhanced by pumping the lysate through a small gauge syringe needle.

▲ **CRITICAL STEP** Immediate freezing of the lysate is essential to complete the lysis process as well as preventing adventitious binding. Additional freeze-thaw cycles should be avoided to prevent protein and RNA degradation.

Antibody coating of protein A/G beads ● TIMING ~15 min

3| At 4 °C, pre-swell protein-A Sepharose beads in NT2 supplemented with 5% BSA to a final ratio of 1:5 for at least 1 h before use. Protein G or A/G Sepharose beads may be substituted depending upon the isotype of the antibody being used.

■ **PAUSE POINT** Pre-swollen beads may be stored for several months at 4 °C when supplemented with 0.02% sodium azide.

4| In a 1.5 ml microcentrifuge tube, add 250–500 μl of protein A-BSA slurry. After a pulse centrifugation this should yield a pelleted bead volume of ~50 μl at the bottom of the microcentrifuge tube.

5| Add antibody to bead slurry and incubate for 2–18 hours, tumbling end over end at 4 °C. The volume of antibody added to the beads is dependent upon antibody titer, but this amount should be more than enough to pull down all available protein being investigated.

■ **PAUSE POINT** This mixture may be stored for several weeks at 4 °C when supplemented with 0.02% sodium azide.

▲ **CRITICAL STEP** In parallel, a control antibody must be used to assess background RNA levels. Typically, this is an isotype-matched antibody or whole normal sera from a matched species. The amount of control antibody should be equal to the amount of immunoprecipitating antibody being used.

6| Immediately before use, wash antibody-coated beads with 1 ml of ice-cold NT2 buffer 4–5 times. To wash, spin down beads by pulsing in an ultracentrifuge at 4 °C, remove liquid with hand pipettor or aspirator and resuspend in ice-cold NT2 buffer by flicking the tube several times with a finger. This washing removes unbound antibody as well as contaminants such as RNases, which may be present in the antibody mixture, and is one of the reasons we pre-bind the antibody to beads.

7| After the final wash, resuspend beads in 850 μ l of ice-cold NT2 buffer. Add 200 units of an RNase inhibitor (5 μ l RNase Out), 2 μ l (to final concentration of 400 μ M) Vanadyl ribonucleoside complexes, 10 μ l of 100 mM DTT and EDTA to 20 mM.

Immunoprecipitation reaction and RNA precipitation ● **TIMING** ~6 h, including binding incubation

8| Thaw mRNP lysate on ice and centrifuge at 15,000*g* for 15 min to clear lysate of large particles. Transfer cleared supernatant to microfuge tube and store on ice. Additionally, pre-clearing of lysate with beads may be used to reduce background, if necessary. This may, however, reduce signal.

9| Add 100 μ l of cleared lysate to antibody mixture prepared in Step 7.

▲ **CRITICAL STEP** This dilution of lysate is important to reduce adventitious binding.

10| Immediately flick tube several times with a finger to mix, and centrifuge briefly at 8,000–10,000*g* to pellet beads. Remove 100 μ l of supernatant to represent total cellular mRNA.

11| Incubate for 4 h at 4 °C tumbling end over end. Alternatively, incubate 2 h at room temperature (18–25 °C) and times as short as 15 min have worked well in some cases.

12| Pellet beads and save supernatant for later analysis if desired. Supernatant may be stored at –20 °C for several months.

13| Wash beads 4–5 times with 1 ml of ice-cold NT2 buffer by pulsing in an ultracentrifuge and removing supernatant with a hand pipettor or an aspirator.

▲ **CRITICAL STEP** Thorough washing is critical for reducing background. NT2 buffer may be supplemented with urea, sodium deoxycholate or SDS to increase stringency depending upon the RNA-binding protein being investigated. All tubes should be kept on ice as much as possible while working quickly during the washing process to reduce degradation.

14| Resuspend the beads in 100 μ l of NT2 buffer. NT2 buffer can also be supplemented with 30 μ g of proteinase K to release the RNP components. Incubate mixture for 30 min at 55 °C, flicking the tube occasionally using a finger.

15| Release the RNP components and isolate the RNA from the immunoprecipitated pellet by adding either Trizol reagent (Invitrogen) or phenol-chloroform-isoamyl alcohol directly to the beads. Precipitate RNA and resuspend in a volume appropriate for subsequent detection method. Addition of glycogen (20 μ g) as a carrier to the precipitation reaction aids in making the RNA pellet more readily visible and aids in recovery of RNA.

16| After release of the RNP components, one can isolate the proteins associated with the complex and submit them for standard mass spectroscopy or other proteomics identification procedures. Such information can be very useful in the functional analysis subsequent to the identification of RNA subsets as described above.

● **TIMING**

Steps 1–2: 1 h

Steps 3–7: 15 min

Steps 8–15: 6 h

? **TROUBLESHOOTING**

If after RNA isolation and analysis there is adequate signal from total RNA but poor or no signal from RNA isolated through RIP-Chip, there are several steps that may be taken to ascertain possible causes. After confirming that there is adequate expression of the protein of interest within the cell, it is necessary to confirm that this protein was recovered via RIP-Chip. To do this, RIP-Chip is performed exactly as described above but stopped before Step 14 (before the addition of proteinase K). The washed beads are resuspended and boiled in Laemmli buffer to recover associated proteins. Western blot analysis is performed using this material to determine if the RBP of interest has been effectively recovered through RIP-Chip.

If the protein is not detected, one possible explanation is inaccessibility of the epitope to which the immunoprecipitating antibody is directed. Therefore, one may use an epitope-tagged RBP for mRNP isolation^{13,14}. Alternatively, antibodies recognizing different epitopes may be used for RIP-Chip. In general, this has not been an issue because most RBPs are present on the surfaces of RNPs and polyclonal antibodies (e.g., many human autoantibodies against RBPs) are presented to the immune systems as RNP complexes^{3,9,30}. Even under conditions in which the RBP or RNP-associated protein is detected in the cell extract, RIP-Chip may need to be further optimized for the specific mRNP being investigated. As noted above,

more stringent washing may be achieved by adding optimally determined concentrations of urea, SDS, deoxycholate or other detergents and chaotropic agents to the wash buffer to reduce the background. If an RNA component of the mRNP is known, one can reverse transcribe the RNA and perform PCR to quickly optimize the RIP wash conditions¹⁴. In this manner, a large number of conditions may be quickly screened to reduce background nonspecific binding. Alternatively, quantitative real-time PCR may be used to discern PCR cycle differences that are not obtainable when using nonquantitative PCR. We have also found that multiprobe RNase protection assays offer a rapid way to optimize RIP^{13,14}. As mentioned above, correct choices of variations of these methods are best served with knowledge of at least certain components of the mRNP. For example, binding of members of the RNA-recognition family (RRM) and Pumilio family of RNA binding proteins are not affected by the presence of urea, whereas other RBPs may be affected adversely. Further caution should be taken to make sure that the antibody or ligand tag are not affected by harsh washing conditions.

ANTICIPATED RESULTS

Depending on the method used to detect RNA targets, the exact results and strategy of global analysis may vary. When dealing with large data sets of putative mRNA targets first identifying the biological function(s) of the RBP with respect to a single or small set of mRNA species can be advantageous. Certain functions may become more readily obvious when looking at the entire list of targets than when focusing on a single gene. This can be accomplished by running the entire list of mRNA targets through gene ontology (GO) programs, such as Panther (<http://www.pantherdb.org>) or Webgestalt (<http://genereg.ornl.gov/webgestalt/>)³⁴, which search annotated GO categories and provide statistical analysis indicating enriched GO categories. In this type of analysis it is important to compare the target mRNAs of the RBP to mRNAs expressed in that cell type rather than to a general global list of mRNAs for that organism. Examples of this type of analysis have been demonstrated in yeast²⁷, *Drosophila*²⁸ and mammalian cells^{13,34,35}.

Furthermore, it is more probable to identify sequence or structural motifs common to an entire set of targets when using large lists of targets than when dealing with only a few targets. These motifs, however, frequently contain significant variability and are often poorly characterized, thus making their identification difficult with small data sets. This type of analysis has been done effectively with Hu proteins^{13,36}, the Fragile X Mental Retardation protein³⁵ and Pumilio proteins^{27,28}.

One of the limitations of the RIP-Chip procedure is that it is often difficult to distinguish 'direct' from 'indirect' RNA binders. Unfortunately, unbiased methods to make this distinction are still less than satisfactory. In general, however, the field has enjoyed abundant success in identifying mRNAs within isolated RNP complexes that can be validated as *bone fide* targets using a variety of methods including: (i) the use of alternative reagents, (ii) multiple cell types, (iii) using wide latitudes of reagent concentration, (iv) using alternative detection methods (e.g., microarrays, QRT-PCR, northern blotting), (v) genetic validation and (vi) noting predictable structural and functional relationships among the mRNA subsets. Therefore as noted above, it is always appropriate to optimize the conditions for preparing lysates and the antibody ligand or epitope tag that function best with the RNP or RBP of interest. As demonstrated and reviewed in several articles, the methods described by Tenenbaum *et al.*, when properly optimized have performed very well for many investigators with minimal alteration^{5,6,13–16,19,27–29,35,36}.

COMPETING INTERESTS STATEMENT The authors declare competing financial interests (see the HTML version of this article for details).

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Corrigendum: RIP-Chip: the isolation and identification of mRNAs, microRNAs and protein components of ribonucleoprotein complexes from cell extracts

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In the version of the article originally published, in the last sentence of the ANTICIPATED RESULTS section, the callout should be to reference 19 instead of 18. The error has been corrected in the HTML and PDF versions of the article.