

RIP-chip analysis of RNAs bound to Chloroplast RBPs

Barkan lab, 2007 (updated 3/13/08)

This is almost, but not quite, identical to the chapter published in *Methods in Molecular Biology*. This has several clarifications to avoid high background on the slides. If you use this protocol in published work, please cite A Barkan, Genome-wide analysis of RNA-protein interactions in plants. (2009) *Methods in Molecular Biology* 553: 13-37.

3.1 Lysate Preparation

Chloroplasts are purified from seedling leaves according to any standard protocol. The chloroplast pellet is lysed by incubation for 15 min on ice in a minimal volume of Hypotonic Lysis Buffer, punctuated with several rounds of vortexing. To complete the lysis, the material is drawn with a syringe through a 21 gauge needle and syringed up and down several times; bubbles should be avoided. Membranes are pelleted by centrifugation in a microfuge (4°C) at the highest setting for 30 min. The supernatant, which contains the stroma and some envelope membranes, is used for the RIP-chip assays (see **Note 1**). The protein concentration of the extract is determined with a Bradford assay (Bio-Rad) and should be between 5 and 20 mg/ml. The extract is flash-frozen and stored in aliquots of ~100 μ l (~1 mg protein) at -80°C. Freeze-thaws prior to immunoprecipitation should be avoided. Each aliquot is used for between two and four RIP-chip assays, performed in parallel. A modification of this method that has succeeded for maize mitochondrial extracts is described in **Note 1**.

3.2 Experimental Design

Our procedure uses spotted microarrays probed simultaneously with differentially-labeled RNAs from the immunoprecipitation pellet and supernatant. Although total input RNA or RNA from the pellet of a mock precipitation can be used as the reference sample, we have obtained better results by using the supernatant RNA as the reference (see ref (**38**)).

A negative control is essential to identify abundant RNAs or “sticky” RNPs that contaminate the immunoprecipitation pellet. An effective control for this purpose uses affinity purified antibody to a different protein, at a similar IgG concentration to that used for the experimental sample. An immunoprecipitation using extract prepared from material that lacks the target antigen but that is otherwise similar to the experimental extract (e.g. from a null mutant, or from genotype-matched cells not expressing the tagged isoform, if a tag is used) is an excellent control, if such material is available.

3.3 Immunoprecipitation

3.3.1. Wash Staph A Cells

Formalin-fixed Staph A cells must be washed thoroughly to remove any dissociated Protein A because this will titrate out the antibody and reduce the yield of the precipitation.

1. Shake the bottle of cells to give a homogeneous suspension. Remove a quantity of cell suspension that is sufficient for several days use. Divide the suspension between several 1.5 ml microfuge tubes; place less than ~ 0.8 ml in each tube because large cell pellets can be difficult to resuspend.
2. Pellet the cells by centrifugation for ~1 min in a microfuge at ~10,000 rpm. Pipet off the supernatant, and replace it with a similar volume of colP buffer. Pipet up and down vigorously to resuspend the cells.
3. Repeat this washing procedure two more times, for a total of three washes. Resuspend the final cell pellet in colP buffer to the initial volume. Combine the aliquots. Store at 4°C for up to 2 days.

3.3.2. Preclear Lysate

The quantity of stromal extract to use for a RIP-chip experiment will depend upon the abundance of the target RNPs. Aliquots of extract containing 0.5 mg of protein have yielded strong RIP-chip signals with a variety of low abundance plastid RBPs (**37-40**). For more abundant RBPs, less lysate should be sufficient. Typically, several immunoprecipitations are performed with each thawed aliquot of lysate. It is convenient to perform these pre-clearing steps before dividing the stroma for subsequent immunoprecipitations.

1. Place sufficient extract for control and experimental immunoprecipitations on ice and thaw slowly. Add RNAsin (Promega) to the thawing stroma (~20 units per 100 μ l stroma) to reduce RNA degradation (see **Note 2**).
2. Centrifuge the extract for 10 min at ~12,000 rpm in a microfuge to remove insoluble particles. THIS STEP IS VERY IMPORTANT TO REDUCE BACKGROUND.
3. Further pre-clear the supernatant with washed IgSorb Staph A cells, as follows. Centrifuge 100 μ l of the washed StaphA cell suspension briefly to pellet the cells. Discard the supernatant. Resuspend the cell pellet in the cleared stromal lysate from step 2 above. Pipet up and down to resuspend the pellet, avoiding bubbles. After 10 min on ice, pellet the cells by centrifugation for ~5 min at ~10,000 rpm in a microfuge (4°C). Carefully pipet the supernatant into new tubes for use in the immunoprecipitation reactions. Reserve a small aliquot (e.g. 1/20th) of this supernatant for immunoblot analysis to assess the success of the immunoprecipitation.

3.3.3 Antibody Binding

1. Add affinity-purified antibody to the lysate. The optimal amount of antibody needs to be determined empirically (see **Note 3**), but will typically be between 2 and 10 μ l.
2. Leave on ice for 1 h with occasional gentle mixing.

3.3.4. Precipitation of Antigen-Antibody Complexes

1. Shake a tube of washed Staph A cells to suspend the cells, and add 100 μ l of the suspension to each immunoprecipitation. Mix gently. (If using crude serum, see **Note 3**).
2. Store on ice for 30 min with occasional gentle mixing.
3. Pellet cells by microcentrifugation at ~10,000 RPM for 1 min.
4. Carefully pipet off the supernatant. Remove 1/10th of the supernatant to a separate tube to be used for immunoblot analysis; the remainder will be used for RNA extraction. Store both tubes of supernatant at -80°C until needed.
5. Resuspend cells thoroughly in ~0.5 ml of coIP buffer, by pipetting up and down. (See **Note 4** for alternative wash buffers used to reduce non-specific binding.) Be sure to disrupt all visible cell clumps.
6. Pellet the suspended cells by microcentrifugation for 1 min (~10,000 RPM). Discard the supernatant.
7. Repeat this washing procedure two more times, for a total of three washes.
8. Resuspend the final washed cell pellet in 250 μ l of Co-IP Buffer. Do not add Mg⁺⁺ to this buffer even if it had been used in the immunoprecipitation.
9. Remove a 25 μ l aliquot of the suspension to a separate tube for the immunoblot analysis described in Step 3.3.6. Pellet the cells in this aliquot by microcentrifugation for 1 min and resuspend the cells in 20 μ l 1.5 X SDS Sample Buffer. Store at -20°C until ready for SDS-PAGE. For long term storage (>2 days), store at -80°C.

3.3.5. RNA Purification

1. Increase the volume of the reserved immunoprecipitation supernatant to match that of the immunoprecipitation pellet sample (~225 μ l) by adding co-IP buffer (lacking Mg^{++}).
2. To disrupt the RNPs, add 25 μ l 10% SDS and 10 μ l 200 mM EDTA to each pellet and supernatant sample.
3. Add 1 μ l GlycoBlue (Ambion) to the pellet sample. The GlycoBlue enhances the recovery of the small amount of RNA in this sample by serving as a carrier and by making it easier to visualize the RNA during the subsequent purification steps.
4. Add ~250 μ l of phenol-chloroform-isoamyl alcohol to each sample. Vortex thoroughly and separate the phases by microcentrifugation at 10,000 rpm for 5-10 min at room temperature.
5. Carefully remove the aqueous phases to new tubes, being sure to avoid any interface material. Aqueous phase left with the organic phase at this point will be recovered during the back-extraction that follows.
6. Back-extract the organic phase and interface by adding 150 μ l of 10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 0.25% SDS. Vortex thoroughly and centrifuge as above. Carefully remove the aqueous phase and combine with the corresponding aqueous phase from the first extraction.
7. Bring the sodium concentration to ~0.3 M by the addition of ~20 μ l 3 M NaAcetate. Add 2.5 volumes of ethanol (~1 ml) to each tube. Vortex. Store at $-20^{\circ}C$ for at least 1 h. The RNA can be stored indefinitely at this step.
8. Pellet the RNA by microcentrifugation at $4^{\circ}C$ at $>12,000$ rpm for 15 min.
9. Carefully pipet off the ethanol. (The RNA pellet from the immunoprecipitation pellet sample should be blue). Rinse the RNA pellets by adding ~500 μ l 70% ethanol, vortexing briefly, and microcentrifuging at ~12,000 rpm for 10 minutes. Pipet off most of the ethanol. Air dry the pellets by inverting the tubes on to clean KimWipes for ~15 min. Alternatively, dry the pellets in a Speed Vac, but be careful not to over-dry the pellets as the RNA may become difficult to resuspend.
10. Resuspend the RNA from the immunoprecipitation pellet sample in 12 μ l RNase-free water. Resuspend the RNA from the immunoprecipitation supernatant sample in 36 μ l RNase-free water. Store at $-80^{\circ}C$ until ready for the labeling reaction.

3.3.6 Immunoblot Analysis to Evaluate the Immunoprecipitation

Before proceeding with RNA labeling, the recovery of the target RBP in the immunoprecipitation should be checked by SDS-PAGE and immunoblot analysis. An equal proportion of the pellet and supernatant material (e.g. 1/10th of each), and a corresponding amount of the starting extract should be analysed. If the RBP comigrates with the IgG heavy chain, then its signal will be obscured with immunoblot detection methods that use an anti-IgG antibody as the secondary antibody. The "One-Step Western Blot Kit" (GenScript) gives excellent results in this situation.

3.4 RNA Labeling

RNAs purified from the pellet and supernatant fractions are differentially labeled with fluorescent dyes by using the Micromax ASAP RNA labeling kit (Perkin-Elmer). This procedure labels the guanosine bases in both RNA and DNA, so the RIP-chip protocol described here can be modified for use with DNA binding proteins (see **Note 5**). We routinely label the pellet RNA with Cy5 and the supernatant RNA with Cy3; an experiment in which chloroplast RNA labeled with each of the two dyes was competitively hybridized to a microarray demonstrated that dye-bias is minimal (**38**).

The oxidation of Cy5 by environmental ozone can severely decrease the fluorescence yield; even though this problem occurs only sporadically, it is safest to routinely take the following precautions. Minimize exposure to ozone by performing all steps possible in a nitrogen environment: Pour a 1" layer of liquid nitrogen into a large styrofoam tub. Place a microfuge tube rack into the tub, positioned so that the tubes will be above and not in contact with the liquid nitrogen. Open all tubes containing Cy5 and do all manipulations of Cy5-containing solutions in this environment. Do not leave tubes in the tub for prolonged periods as their contents will freeze. We suspect that the most ozone-sensitive step is the first one, when the Cy5 stock solution is opened and the Cy5 reagent is added to the RNA.

One-half of the RNA recovered from the pellet and one-sixth of the RNA recovered from the supernatant is labeled and used for hybridization. A smaller fraction of the supernatant sample is used in order to reduce saturation of array fragments complementary to the highly abundant rRNAs and tRNAs.

1. Set heating blocks to 85°C and 60°C. Place a plastic tub full of water into an oven or water bath set to 58°C for microarray hybridization. Set a Slide Warmer (LabLine Instruments) to ~55°C.
2. Pipet 6 µl of each RNA into separate 0.5 ml microfuge tubes. To each tube add 3 µl of the Labeling Buffer supplied with the MicroMax ASAP kit (see **Note 6**).
3. Add 1 µl of the appropriate fluorescent labeling reagent supplied with the MicroMax kit to each sample. Cy dyes are light sensitive so return them to their dark, refrigerated storage area immediately after use.
4. Place the reactions in the 85°C heat block for 15 min (see **Note 7**).
5. Transfer tubes to ice and add 2.5 µl ASAP Stop Solution.
6. The RNA is separated from free dye by purification with a Qiaquick Nucleotide Removal Kit (Qiagen). Add 250 µl Buffer PN supplied with the Qiagen kit, at room temperature. Transfer the mixture to a Qiaquick spin column. Let stand for 1 min at room temp. Centrifuge for 1 min at 10,000 rpm in a microfuge. Discard the flow through (which should contain only free label).
7. Wash the column by adding 500 µl of Buffer PE supplied with the column. Centrifuge for 1 min at 10,000 rpm in a microfuge at room temperature. Remove the flow through and centrifuge the column for one additional minute to remove excess ethanol.
8. Elute the RNA from the column by transferring the column to a new tube, adding 40 µl 5 mM Tris-HCl pH 8.3 (see **Note 8**), and centrifuging for 1 min at 10,000 rpm. Repeat the elution step by adding an additional 40 µl of 5 mM Tris pH 8.3 to the same column in the same tube and centrifuging for 1 min at 10,000 rpm. The eluted supernatant RNA should be visibly pink due to the coupled Cy3, but some pink dye is typically retained in the column. The eluted pellet sample is not generally blue to the eye.
9. Concentrate the eluted RNA samples in a Speed Vac (without heat) until ~5 µl remains in each tube. It is important not to dry the RNA to completion. This step takes ~30 min. We use the labeled RNA immediately for hybridization, but instructions for long term storage are provided by the manufacturer of the labeling reagents.

3.5 Microarray Hybridization

Hybridization is performed in a Microarray Hybridization Chamber (Corning #2551). A constant temperature is maintained during hybridization by submerging the sealed Microarray Hybridization Chamber in a water bath in a sealed plastic container. Several hours in advance, pre-warm the water bath in the plastic container by placing it in a hybridization oven or water bath set to 58°C.

1. Warm Hybridization Buffer III supplied with the MicroMax kit to 60°C in a heat block. Add 30 μ l of the warm Hybridization Buffer III to each labeled RNA sample (~5 μ l each).
2. Combine the pellet and supernatant RNA samples (total of ~70 μ l). Heat for 3-4 min at 60 °C.

During this incubation:

- (i) Place a LifterSlip Premium Printed Coverslip (Erie Scientific) on top of the microarray, being careful to place the tape side down so that there is a gap between the slide and coverslip.
 - (ii) Prewarm the microarray slide/coverslip assembly and the microarray hybridization chamber by placing them onto the slide warmer (set to ~55°C).
3. Centrifuge the warmed RNA samples very briefly in a microfuge at room temperature to pellet condensation. Minimize cooling of the sample (see **Note 9**). Immediately pipet the warm RNA onto the pre-warmed array by placing the pipet tip next to the opening at either end of the coverslip. Pipet slowly, checking that the sample is being drawn under the coverslip by capillary action. Place the slide into the microarray hybridization chamber. Pipet ~15 μ l of 3X SSC into each of the two wells in the microarray hybridization chamber; this is essential to keep the array from dehydrating.
 4. Seal the chamber and place it in the 58°C water bath inside the plastic container. Seal the container and place it in the 58°C oven or water bath. Incubate overnight.

3.6 Microarray Washing

1. Fill a slide staining dish with 0.01% SDS, 0.5X SSC (room temperature).
2. Remove the microarray slide/coverslip assembly from the hybridization chamber. Place it in the slide holder that accompanies the slide-staining dish. Do NOT remove the coverslip manually. Instead, dunk the slide holder gently in the solution until the cover slip passively detaches from the slide. The cover slip should fall to the bottom of the dish.
3. Wash 1: Place the staining dish on a rotary shaker and shake gently (~50 rpm) for 15 min.
4. Wash 2: Transfer the slide in its holder to a new staining dish, containing 0.01% SDS, 0.06X SSC. Shake at ~60 rpm for 15 min.
5. Wash 3: Transfer the slide in its holder to a new staining dish, containing: 0.06 X SSC. Shake at ~60 rpm for 15 min.
6. Dry the slide by centrifuging the slide in the slide holder in a table-top centrifuge for 3 min at 550 rpm. It is important to centrifuge the slide immediately; failure to keep the slide immersed until immediately before the centrifugation can result in high background.

3.7 Microarray Scanning and Data Analysis

Scan slides as soon as possible as the fluorescent signals diminish over time. It is useful to scan at several laser intensities: lower intensities reduce saturation for highly abundant RNAs (e.g. rRNAs and tRNAs) whereas higher intensities can be important to detect low abundance RNAs. Scans at 532 nm (which elicits green fluorescence from Cy3) are typically performed at a PMT gain between 400 and 550. Scans at 635 nm (which elicits red fluorescence from Cy5) are typically performed at a PMT gain between 450 and 650. Store the slides in a 50 ml conical centrifuge tube covered in foil at 4 °C. Slides can be rescanned several times within a few days with only a small loss of signal. The specifics of the scanning and data analysis procedures will vary with the array platform and facilities available. We import the data into GenePix Pro 6 (Molecular Devices) and filter out low quality spots as described in Schmitz-Linneweber et al. (38).

3.8 Validation of Results by Slot-blot Hybridization

Slot-blot hybridization can be used to validate positives to emerge from the RIP-chip data (*see Note 10*), and to pinpoint the RNA sequences associated with an RBP to greater resolution than is generally possible from the microarray data alone (*see Note 11*). RNAs purified from experimental and control immunoprecipitation pellets and supernatants are applied to slot blots and analyzed by hybridization to probes that correspond to array positives. If the number of putative positives is small, each of them can be validated in this way. In large-scale studies, a sampling of the positives should be validated.

1. Perform immunoprecipitations and extract RNA from the pellets and supernatants as for the RIP-chip assays.
2. Resuspend the RNA purified from each pellet and supernatant in 1200 μ l 2X SSPE. 100 μ l of the resuspended RNA samples will be applied to each slot. Heat the RNA to 70 °C for ~ 10 min, while setting up the slot blotter.
3. Cut nylon hybridization membrane (MagnaNylon) to fit the slot-blot manifold and prewet it in 1X SSPE. Place the membrane into the slot blotter and place under vacuum for ~ 1 minute, to dry the membrane slightly.
4. Pipet 100 μ l of each RNA sample into a separate slot while under vacuum. Allow the vacuum to pull the solution through the membrane. For each validation test, the following samples should be included: i) Pellet and supernatant RNAs for the experimental antibody; (ii) Pellet and supernatant RNAs for a negative control antibody; (iii) Total RNA extracted from an amount of stroma equivalent to that used for each immunoprecipitation. Store unused RNA at -80°C .
5. Remove the nylon membrane from the slot blot apparatus and place the side to which the RNA was applied face up on Whatman 3MM paper soaked with 1X SSPE. Crosslink the RNA to the membrane in a UV crosslinker (e.g. Stratalinker in "optimal cross-link" mode.)
6. Air dry the membrane. Prehybridize and hybridize the membrane using probes corresponding to each validation test, using standard conditions for RNA gel blots.

4. Notes

1. All of the chloroplast RBPs we have studied are in the soluble fraction. However, if the RNP of interest is membrane-associated, it will need to be stripped from the membrane or the membrane will need to be solubilized with non-ionic detergent prior to immunoprecipitation. We have begun to modify this method for maize mitochondrial RBPs. Most mitochondrial RNAs and ribosomes pellet with the membrane fraction after organelle lysis. We have obtained interpretable RIP-chip data with a mitochondrial lysate generated by solubilization of the mitochondrial pellet with 1% NP-40. However, further optimization of this method is likely to be useful.
2. To increase the resolution of the assay so that the site of RBP binding within a large RNA ligand can be pinpointed, ribonuclease inhibitors should be omitted. This allows endogenous ribonucleases to reduce the size of the coimmunoprecipitated RNA molecules so that tethering of sequences distant from the binding site is reduced. We were able to pinpoint the binding sites of one RBP to within ~ 100 nucleotides by probing the RNAs coimmunoprecipitated in this manner with tiled 70-mer oligonucleotides (**38**). Hybridization of the coimmunoprecipitated RNA to an oligonucleotide tiling microarray or sequencing of the coimmunoprecipitated RNAs are alternative methods to identify enrichment peaks to high resolution.
3. The amount of antibody needs to be determined empirically. Antibodies that have been affinity purified against the antigen are ideal because the majority of the IgGs will be directed against the protein of interest, and the low quantity of IgGs ensures their quantitative precipitation by the Staph A cells. Titrations should be performed to determine how much

antibody is needed to recover most of the target RBP in the immunoprecipitation pellet; 5 μ l of affinity purified antibody is a good starting point. Crude serum can sometimes be used successfully. However, the quantity of Staph A cells may have to be increased to ensure quantitative binding of the IgGs, and the abundant IgGs may complicate the interpretation of immunoblot tests of immunoprecipitation efficiency.

4. The stringency of the immunoprecipitation can be increased by performing the first wash in a different buffer (e.g. colIP buffer supplemented with 500 mM NaCl or with 0.5% deoxycholate). However, these treatments may disrupt the RNPs of interest.
5. The Micromax ASAP RNA Labeling Kit modifies the N7 position of guanosine with the fluorescent dye in both DNA and RNA. DNA contributes only a small fraction of the total signal except for sequences that are not transcribed or that yield very low abundance RNAs. Thus, the contribution of DNA to the signal can generally be ignored. However, some proteins are bound to both RNA and DNA in chloroplast lysate (unpublished results), and in some cases, it may be desirable to use this method to identify the binding site of a DNA binding protein. To ensure that the signal arises from only RNA or only DNA, DNA or RNA can be removed from the immunoprecipitated material with DNase or alkali hydrolysis, respectively. RNase A is less effective than alkali hydrolysis to eliminate the RNA because rRNAs are highly abundant and structured, so they are resistant to ribonuclease digestion.

Alkali hydrolysis is performed as follows. Resuspend the nucleic acids recovered from the immunoprecipitation pellet and supernatant each in 40 μ l of dH₂O. Add 10 μ l 1 N NaOH. Incubate at 70 °C for 30-45 min. Neutralize the pH with the addition of 2.1 μ l 4.8 N HCl and 2.5 μ l 1 M Tris-HCl pH 7.5. Precipitate the DNA by adding 150 μ l EtOH, placing the tubes at -20°C for at least 30 min, and microcentrifugation for 20 min. Resuspend each DNA pellet in 12 μ l dH₂O and label this material using the MicroMax kit as described above for RNA. If the hydrolysis was successful, the fluorescence associated with rDNA fragments will be similar to that from other fragments.

Elimination of signal arising from DNA is not necessary in most cases. However, if it is suspected that the bait protein interacts with both DNA and RNA, then this step can clarify the source of the fluorescent signal. After extracting nucleic acids from the immunoprecipitation pellet and supernatant, resuspend each sample in 48 μ l dH₂O. Add 1 μ l RNasin (Promega), 6 μ l 10X RQ1 DNase Buffer and 2 μ l RQ1 DNase, RNase free (1unit/ μ l) (Promega, # M6101). Incubate at 37 °C for 30 min. Add 140 μ l dH₂O, 20 μ l 10% SDS, 5 μ l 0.2 M EDTA, and 4 μ l 5 M NaCl. Phenol-chloroform extract and back extract as described in Step 3.3.5. Add 1 μ l GlycoBlue to the pellet sample and ethanol precipitate as above. Resuspend the RNAs derived from the immunoprecipitation pellet and supernatant in 12 μ l and 36 μ l of water, respectively.

6. The volume of RNA in the labeling reactions can be varied. However, the 10 μ l reaction must include at least 2 μ l of Labeling Buffer.
7. This incubation time determines the proportion of the guanosines that will be labeled. Too short an incubation results in poor labeling, but too long an incubation yields RNA that is so heavily modified that it hybridizes poorly. A 15 min incubation is reported by the vendor to be optimal for an mRNA of "average" length. However, for short RNA fragments, it may be possible to further optimize this step.
8. A pH above 8 is critical for elution of RNA from the column. H₂O can be effective for elution, but it is prudent to add a low concentration of buffer to control the pH.
9. For DNA and highly-structured RNAs, reheating the sample to 80°C for 30 seconds may better denature the nucleic acids. This additional heating also reduces viscosity, allowing the sample to slide more smoothly under the coverslip on the microarray. The dyes are heat-stable so this additional heating is not detrimental.

10. Real-time PCR or any other quantitative assay could be used as an alternative.
11. To pinpoint sites of RNA interaction at selected loci, coimmunoprecipitated RNA can be applied to replicate slot blots and hybridized with tiled oligonucleotides to detect peaks of enrichment within a large RNA molecule (**38**). For such experiments, ribonuclease inhibitors should not be added to the immunoprecipitation reaction.