RNA Immunoprecipitation for Determining RNA-Protein Associations In Vivo

Chromatin immunoprecipitation (ChIP; UNIT 21.3) is a powerful and widely applied technique for detecting the association of individual proteins with specific genomic regions in vivo. RNA immunoprecipitation (RIP) is very similar, but, as the name implies, this latter method focuses on protein-RNA interactions. In RIP (as in ChIP), live cells are treated with formaldehyde to generate protein-protein, protein-DNA, and protein-RNA cross-links between molecules in close proximity. A whole-cell extract is prepared in the presence of RNase inhibitors to maintain the integrity of RNA, and the cross-linked nucleic acids are sheared by sonication to reduce average fragment size to \( \sim 500 \) bases. The extract is then treated with DNase I to remove DNA, and the resulting material is immunoprecipitated with an antibody against the protein of interest. RNA sequences that directly or indirectly cross-link with a given protein are selectively enriched in the immunoprecipitated sample. Reversal of the formaldehyde cross-linking by heating permits the recovery and quantitative analysis of the immunoprecipitated RNA by reverse transcription (RT) PCR analysis. The amounts of a specific RNA sequence in control and immunoprecipitated samples are determined individually by quantitative RT-PCR. The fold enrichment of certain RNA sequences (e.g., presumed binding sites) relative to other sequences (e.g., presumed nonbinding sites) provides quantitative information about the relative level of association of a given protein with different regions. Protein association with specific RNA regions can be assayed under a variety of conditions (e.g., environmental change or cell-cycle status) and/or in wild-type versus mutant strains. Furthermore, as formaldehyde inactivates cellular enzymes essentially immediately upon addition to cells, RIP provides snapshots of protein-RNA interactions at a particular time point, and is therefore useful for kinetic analysis of events occurring on RNA in vivo. In addition, like ChIP, RIP can theoretically be combined with microarray technology to identify the location of specific proteins on a “genome”-wide basis (see Commentary). This unit describes the RIP protocol for cells of the baker’s yeast Saccharomyces cerevisiae (see Basic Protocol). It is also applicable to other organisms, although some organism-specific modifications related to cell lysis and sonication will probably be necessary.

RNA IMMUNOPRECIPITATION IN YEAST CELLS

In this RIP protocol, Saccharomyces cerevisiae cells are cross-linked with formaldehyde, then harvested and sonicated to release and minimize the length of nucleic acid molecules. DNA is removed by treatment with DNase I, and the RNA is protected by the addition of RNase inhibitor throughout. The protein-RNA molecules are then immunoprecipitated and purified, the cross-links are reversed, and the resulting RNA is analyzed by RT-PCR.

Materials

- Saccharomyces cerevisiae cells to be studied (see Chapter 13)
- 37% formaldehyde (store up to 1 year at room temperature)
- 2 M glycine, sterilized by autoclaving
- Tris-buffered saline (TBS; APPENDIX 2), ice cold
- FA lysis buffer (see recipe), ice cold and room temperature
- 40 U/µl RNasin (Promega)
- \( \sim 0.5 \)–mm-diameter silica-zirconia (preferably BioSpec) or glass beads
- Ice/salt mixture in beaker for cooling
- MgCl\(_2\)
RNA Immuno-precipitation

27.4.2

**CaCl\(_2\)**
20 mg/ml RNase-free DNase I (Sigma)
0.5 M EDTA (**APPENDIX 2**)

Primary antibody against protein or epitope of interest
50% (v/v) protein A–Sepharose beads (Amersham Biosciences or equivalent) in
FA lysis buffer containing 1 mg/ml BSA
FA lysis buffer (see recipe) containing 1 mg/ml BSA
FA500 (see recipe)
LiCl wash solution (see recipe)
TE/100 mM NaCl (see recipe)
ChIP elution buffer (see recipe)
5 M NaCl, sterilized by autoclaving
20 mg/ml proteinase K (Roche) in TBS/50% glycerol (store up to 1 year at −20°C)
Acid-equilibrated 5:1 phenol/chloroform, pH 4.7 (Sigma, cat. no. P1944)
Phase Lock Gel, Heavy (Eppendorf)
3 M sodium acetate, pH 5.5 (**APPENDIX 2**)
Glycogen
Absolute ethanol, ice cold
70% ethanol
TE buffer, pH 7.5 (**APPENDIX 2**)
Titan One- Tube RT-PCR kit (Roche)
6% acrylamide/bisacrylamide (19:1) nondenaturing PAGE gel prepared in TBE
buffer (see **UNIT 2.7**)
SYBR Green (Molecular Probes)
500-ml Erlenmeyer flask
Platform rocker
50-ml conical centrifuge tubes (e.g., Falcon)
Refrigerated centrifuge
1.5-ml (nonstick) microcentrifuge tubes, certified RNase-free
FastPrep benchtop cell disruptor (Qbiogene)
Hypodermic needle
2-ml microcentrifuge tubes
15-ml conical polypropylene centrifuge tubes, disposable (e.g., Falcon)
Sonicator with microtip probe (e.g., Branson Sonifier 250)
End-over-end rotator
Spin-X microcentrifuge tube filters (Corning, available, e.g., from Sigma)
42°C (optional) and 65°C water baths
Thermal cycler

Additional reagents and equipment for growth of *Saccharomyces cerevisiae*
cultures (**UNITS 13.1 & 13.2**), determining chromatin fragment size (**UNIT 21.3**),
phenol/chloroform extraction and ethanol precipitation (**UNIT 2.1A**), primer design
for ChIP experiments (**UNIT 21.3**), oligonucleotide synthesis (**UNIT 2.11**), PCR (**UNITS
15.1 & 15.7**), nondenaturing polyacrylamide gel electrophoresis (**UNIT 2.7**), and
agarose gel electrophoresis and ethidium bromide staining of gels (**UNIT 2.5A**)

**NOTE:** Remember that this procedure is concerned with detecting RNA; therefore, great
care has to be taken to avoid its degradation during handling. After harvesting of cells,
use RNase-free tubes as indicated, pipet tips with aerosol-barrier filters, and solutions
prepared with nuclease-free water (Ambion). Keep samples on ice.

**Cross-link protein-RNA complexes in vivo**

1. In a 500-ml Erlenmeyer flask, grow 100 ml of yeast cell culture (**UNIT 13.2**) to mid-
logarithmic phase in the appropriate medium (**UNIT 13.1**). Measure the cell density
(**UNIT 13.2**) before formaldehyde fixation.
A concentration of $0.5–2 \times 10^7$ yeast cells/ml is usually adequate. The total volume of culture can be reduced (50 ml is a reasonable minimum) or increased depending on need. Typically, 2 to 10 ml of yeast culture ($0.2–1 \times 10^8$ cells) are used for an individual immunoprecipitation. A larger volume permits multiple immunoprecipitations from the same cells, which is particularly useful for experiments involving the analysis of multiple factors or for carrying out independent immunoprecipitations involving the same factor for data reproducibility.

2. Add 2.7 ml of 37% formaldehyde (for a final concentration of 1%). Cross-link by incubating 15 to 20 min at room temperature, occasionally swirling the flask or shaking slowly on a platform rocker.

   CAUTION: Keep cultures covered or work in a fume hood to avoid noxious formaldehyde fumes.

3. Add 10 ml of sterile 2 M glycine (for a final concentration of $\sim0.2$ M) and incubate an additional 5 min at room temperature.

   Glycine stops the cross-linking by reacting with formaldehyde.

### Harvest cells

4. Transfer cells to a 50-ml centrifuge tube and centrifuge 5 min at $2500 \times g, 4^\circ C$. Discard supernatant into a chemical waste container and resuspend pellet in 50 ml ice-cold TBS. Repeat once.

5. Centrifuge cells a third time for 5 min at $2500 \times g, 4^\circ C$, and discard supernatant. Resuspend cells in 0.5 ml ice-cold FA lysis buffer and transfer to a nonstick, RNase-free microcentrifuge tube.

### Lyse cells

6. Microcentrifuge cell suspension 10 sec at maximum speed, $4^\circ C$, and discard the supernatant. Resuspend the cell pellet in 400 µl ice-cold FA lysis buffer. Add 400 µl of dry, solid silica-zirconia or glass beads.

   The cells can remain on ice for a few hours while other samples are being collected, so that all samples may be processed as a group from this point onward. Alternatively, the cells may be frozen in liquid nitrogen or a dry ice/ethanol bath and stored up to several months at $-80^\circ C$. This is particularly helpful if multiple samples are being generated during a time-course experiment. If cells are frozen, they must be thawed on ice before continuing with the procedure.

7. Lyse cells by applying a FastPrep cell disruptor four to five times for 30 sec each at speed 5.5 (total breakage time 2 to 3 min), removing the sample, and incubating 1 min in an ice-water bath after each treatment.

   Alternatively, cell extracts can be prepared using a Mini Bead Beater (BioSpec) or by vortexing, as described in UNIT 21.3.

8. Puncture a hole in the bottom of the tube with a hypodermic needle, place the tube into a 2-ml microcentrifuge tube, and microcentrifuge 1 min at 5000 rpm.

9. Transfer liquid to a 15-ml conical polypropylene centrifuge tube and add 1 ml FA lysis buffer.

### Shear nucleic acids

10. Place the tube in an ice/salt mix in a beaker to maintain temperature. Holding the microtip of a Branson Digital Sonifier near the bottom of the tube to prevent foaming, sonicate the sample for one or two cycles at 50% amplitude for 15 sec. Keep the sample immersed in the ice/salt bath between and during sonications; leave the samples in the ice/salt bath to cool for at least 2 min after each sonication cycle.
Take great care that the sample does not get too hot, as this may reduce the efficiency of subsequent steps.

If a different sonication device is used, empirically determine the conditions necessary to achieve the desired level of DNA shearing.

11. Add the following to 500 µl of the sonicated extract:

- MgCl₂ to 25 mM final
- CaCl₂ to 5 mM final
- 3 µl of 40 U/µl RNasin
- 6 µl of 20 mg/ml RNase-free DNase I.

Incubate at 37°C for 15 min.

12. Stop the reaction by adding 20 µl of 0.5 M EDTA (for a final concentration of 20 mM). Microcentrifuge 5 min at maximum speed and retain the supernatant. Store in aliquots (stable for many weeks) at –70°C.

These steps remove DNA so that the subsequent precipitation and detection steps focus on protein associated with RNA only. The resulting supernatant constitutes the input sample for the subsequent immunoprecipitation. The frozen aliquots are stable for many weeks when stored at –70°C and are suitable for immunoprecipitation.

13. Check RNA fragment size by electrophoresis on a polyacrylamide gel (see UNIT 21.3 for checking fragment size).

RNA pieces should be between 100 to 1000 bases, with an average length of 300 to 500 bases. It is important to shear RNA down to an average length of 400 to 500 bases. Longer fragments will increase the background and decrease the resolution of the region where the protein associates.

Immunoprecipitate

14. Dilute extract equivalent to 0.2–1 × 10⁸ cells (typically 50 to 100 µl of the extract from step 12) to 250 µl with FA lysis buffer containing RNasin. To this diluted extract, add 1 to 5 µl primary antibody against the protein or epitope of interest and incubate overnight on an end-over-end rotator at 4°C.

The actual amount and concentration of the individual antibody has to be empirically determined and can vary considerably. The immunoprecipitation conditions can be varied (e.g., with respect to time, temperature, salt concentration, and/or presence of detergents) if necessary.

15. Microcentrifuge 15 min at maximum speed, 4°C, then transfer the supernatant to a new tube.

16. Add 20 µl of a 50% slurry of protein A–Sepharose beads (equilibrated in FA lysis buffer containing 1 mg/ml BSA). Incubate 1 to 2 hr at 4°C.

Protein A–Sepharose beads are used here because they work well with most monoclonal antibodies derived from mouse and polyclonal sera derived from rabbit. In some cases, the use of other beads (e.g., protein G–Sepharose) may improve binding of some antibodies, including rat IgG.

Wash beads

17. Microcentrifuge 2 min at 3000 rpm, 4°C, and remove supernatant. Resuspend beads in 700 µl FA lysis buffer, room temperature, and transfer mixture into a Spin-X centrifuge tube filter.

The use of Spin-X filters aids in the recovery of the beads after washes and results in better uniformity between different samples. The procedure is also substantially faster with the filters, particularly when multiple samples are processed simultaneously. Alternatively,
conventional microcentrifuge tubes can be used for the washes, and the supernatant can be carefully aspirated after each spin.

18. Place the filter into a 1.5-ml microcentrifuge tube. Mix sample 3 min on an end-over-end rotator, then microcentrifuge 2 min at 3000 rpm, room temperature, and discard the flow-through liquid at the bottom of the tube.

19. Add 700 μl FA lysis buffer, room temperature, to the beads in the filter and repeat the procedures described in step 18 for a second wash.

20. Wash beads successively, each time using the technique described in step 18, with the following solutions:

- 0.7 ml FA lysis buffer
- 1 ml FA500
- 0.7 ml LiCl wash
- 0.7 ml TE/100 mM NaCl.

*For some polyclonal antibodies, the more stringent washes in this step result in a cleaner signal, while gentle washes alone frequently lead to an unacceptably high background. For other antibodies, repeated washes with FA lysis buffer alone, which are gentler, might be more appropriate.*

**Elute protein-RNA from beads**

21. Place filter unit containing the beads into a new 1.5-ml microcentrifuge tube and add 75 μl of ChIP elution buffer. Gently pipet up and down two or three times in order to dislodge beads from the filter. Incubate 10 min in a 37°C water bath.

A water bath is used instead of other heating apparatus in order to improve heat transfer.

22. Microcentrifuge beads 2 min at 3000 rpm, room temperature. Transfer eluate to another tube. Repeat elution with another 75 μl of ChIP elution buffer. Pool eluates and discard filter with beads.

**Reverse cross-links and purify RNA**

23. Add 6 μl of 5 M NaCl (for final concentration of ~200 mM) together with 20 μg proteinase K (from 20 mg/ml stock). Incubate at 42°C for 1 hr (optional), then at 65°C for 1 hr.

*The incubation at 42°C allows for proteinase K digestion of cross-linked polypeptides, while the 65°C incubation results in a reversal of the formaldehyde cross-links. Note that, because fractions (especially nonprecipitated inputs) contain significant amounts of DNase I, complete inactivation of the enzyme in this step (by digestion) is essential for successful RT-PCR.*

24. Add 100 μl nuclease-free water to the 150 μl of RNA, then add an equal volume (250 μl) of acid-equilibrated 5:1 phenol/chloroform, pH 4.7. Use Phase Lock Gel, Heavy, as recommended by the manufacturer for quick separation of the layers.

*See UNIT 2.1A for additional discussion of DNA extraction and precipitation.*

25. Transfer the resulting aqueous layer to a new tube and add 25 μl 3 M sodium acetate, pH 5.5, 20 μg glycogen, and 625 μl ice-cold absolute ethanol. Leave at –80°C for 1 to 2 hr to allow the RNA to precipitate.

26. Microcentrifuge 30 min at maximum speed, 4°C, and remove the supernatant. Wash precipitate by adding 500 μl ice-cold 70% ethanol and microcentrifuging 5 min at maximum speed, 4°C. Discard supernatant and allow pellet to air dry.
27. For storage, and to help avoid degradation of the resulting RNA, redissolve the pellet in 200 µl TE buffer, pH 7.5, then add 2.5 vol of absolute ethanol, but no salt.

This mix can be stored for weeks at −80°C.

28. Remove an appropriate aliquot of the RNA solution (typically 10 to 50 µl). Add 1 to 5 µl of 3 M sodium acetate, pH 5.5, for a final concentration of ~0.3 M, then leave the mixture on dry ice for about 20 min to precipitate RNA. Microcentrifuge and wash pellet with 70% ice-cold ethanol and dry. Redissolve pellet in 5 µl of TE buffer, pH 7.5.

Perform quantitative RT-PCR and analyze products

29. Design primer pairs for the desired RNA regions to be examined (see UNIT 21.3) and synthesize the primer oligonucleotides (UNIT 2.11).

30. Perform RT-PCR using the Titan kit (also see UNITS 15.1 & 15.7), scaling reactions down to a final volume of 25 µl but otherwise following the manufacturers instructions. Use the following RT-PCR program:

<table>
<thead>
<tr>
<th>Initial step:</th>
<th>30 min</th>
<th>50°C</th>
<th>(reverse transcription)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>93°C</td>
<td>(denaturation)</td>
<td></td>
</tr>
<tr>
<td>36 cycles:</td>
<td>20 sec</td>
<td>93°C</td>
<td>(denaturation)</td>
</tr>
<tr>
<td>30 sec</td>
<td>53°C</td>
<td>(annealing)</td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>68°C</td>
<td>(extension)</td>
<td></td>
</tr>
<tr>
<td>Final step:</td>
<td>7 min</td>
<td>68°C</td>
<td>(final extension).</td>
</tr>
</tbody>
</table>

The Titan one-step kit incorporates reverse transcription and PCR into a single reaction.

For a typical measurement, input RNA is tested along with immunoprecipitated samples. Different dilutions of both input and immunoprecipitated material are tested to ensure that the RT-PCR reactions are in the linear range. See Critical Parameters and Troubleshooting for additional discussion.

It is crucial to include a control reaction in which the RT-PCR step is performed without adding reverse transcriptase, to ensure that the final product is due to RNA rather than contaminating DNA.

31. Separate PCR samples on a 6% acrylamide/bisacrylamide gel (UNIT 2.7) and visualize by staining with SYBR Green at a 1/10,000 dilution.

SYBR Green staining is typically used, although similar results can be obtained by using 32P-labeled nucleotides for the RT-PCR reaction. Gels containing radiolabeled PCR products are analyzed by autoradiography or phosphor imaging (APPENDIX 3A).

ALTERNATE PROTOCOL

ANALYSIS BY REAL-TIME PCR

For real-time PCR analysis, RIP is performed exactly as described in the Basic Protocol, through step 28, whereupon one-step quantitative real-time RT-PCR reactions are carried out using the ABI Prism 7000 Sequence Detection System as described below. For basic considerations, see UNIT 21.3.

Additional Materials (also see Basic Protocol)

2× SYBR Green QPCR mix (Abgene AB-1162; contains DNA polymerase)
Multiscribe reverse transcriptase (Applied Biosystems)
ABI Prism 7000 Sequence Detection System, or equivalent
SYBR Green QPCR mix (Abgene AB-1162)
Software for analyzing PCR primers and products

1. Perform RNA immunoprecipitation and purify RNA (see Basic Protocol, steps 1 to 28).
2. Design primer pairs for the desired RNA regions to be examined (see Unit 21.3) and synthesize the oligonucleotides (Unit 2.11).

3. Set up the following reactions for PCR in a volume of 30 µl:

- 15 µl 2× SYBR Green QPCR mix
- 0.1 µl 40 U/µl RNasin
- 0.15 µl Multiscribe reverse transcriptase
- 10 pmol each primer
- Template RNA (see Basic Protocol, step 28)
- H₂O to 30 µl.

4. Carry out RT-PCR using the following cycling program:

- First step: 30 min 50°C (reverse transcription)
- 15 min 95°C (activation)
- 40 cycles: 15 sec 95°C (denaturation)
- 60 sec 60°C (annealing/extension).

5. Quantitate the relative amount of PCR products using appropriate software for the accompanying instrument (also see Unit 21.3).

6. Calculate the apparent immunoprecipitation efficiency for a specific fragment by dividing the amount of RT-PCR product obtained in the immunoprecipitated sample by the amount obtained from the input RNA (see Unit 21.3).

**REAGENTS AND SOLUTIONS**

*Use nuclease-free, double-distilled water in all recipes and protocol steps. For common stock solutions, see Appendix 2; for suppliers, see Appendix 4.*

**ChIP elution buffer**

- 100 mM Tris·Cl, pH 8 (Appendix 2)
- 10 mM EDTA
- 1% (w/v) SDS

Autoclave

Store up to 1 year at room temperature

Just before use, add 40 U RNasin (Promega) per ml buffer

**FA500**

- 50 mM HEPES, pH 7.5
- 500 mM NaCl
- 1 mM EDTA
- 1% (v/v) Triton X-100
- 0.1% (w/v) sodium deoxycholate

Autoclave

Store up to 2 months at 4°C

Just before use, add 40 U RNasin (Promega) per ml buffer

**FA lysis buffer**

- 50 mM HEPES (adjust pH to 7.5 with KOH)
- 140 mM NaCl
- 1 mM EDTA
- 1% (v/v) Triton X-100
- 0.1% (w/v) sodium deoxycholate
Autoclave
Store up to 1 year at room temperature
Just before use, add 100× protease inhibitors (see recipe) to 1× final, and add 40 U RNasin (Promega) per ml buffer

**LiCl wash**
10 mM Tris-Cl, pH 8 (*APPENDIX 2*)
250 mM LiCl
0.5% (v/v) NP-40
0.1% (w/v) sodium deoxycholate
1 mM EDTA
Autoclave
Store up to 2 months at 4°C
Just before use, add 40 U RNasin (Promega) per ml buffer

**Protease inhibitor stock solutions, 100×**
Dissolve the following in 50 ml of 100% ethanol:
1.42 mg leupeptin
6.85 mg pepstatin A
0.85 mg phenylmethylsulfonyl fluoride (PMSF)
1.65 mg benzamidine
Store up to 6 months at −20°C

**TE/100 mM NaCl**
10 mM Tris-Cl, pH 8 (*APPENDIX 2*)
1 mM EDTA
100 mM NaCl
Store up to 2 months at 4°C

**COMMENTARY**

**Background Information**
RNA immunoprecipitation (RIP) is, in many ways, a natural and direct extension of chromatin immunoprecipitation (ChIP). Much valuable background information can be found in *UNIT 21.3*, which should be consulted before attempting the procedures described in this unit. RIP has been used for studying the interaction of proteins with RNA by Gilbert et al. (2004) and Hurt et al. (2004), based on the idea of modifying the ChIP technique and using fairly similar protocols. The paper by Gilbert et al. (2004) forms the basis for the protocols described here. In these papers, the RNA interactions of transcription-related proteins were studied, but other papers have used RIP or similar RNA immunoprecipitation techniques to study the interaction, e.g., of the basic RNAi machinery with noncoding centromeric RNA (Motamedi et al., 2004) or the interaction of proteins with tRNA (Huang et al., 2005).

The procedure described here was developed for immunoprecipitation of myc epitope–tagged protein in yeast (Gilbert et al., 2004). However, as in ChIP, any specific antibody directed against a protein of interest can be used. The key difference between RIP and ChIP is the use of extensive DNase I digestion of the crude extracts prior to immunoprecipitation. Importantly, the inclusion or omission of this step makes it possible to perform both ChIP and RIP on the same starting cell population, which can often be desirable. Moreover, in RIP, the final detection step for the coprecipitated nucleic acid is RT-PCR, rather than PCR. Hence, although as with ChIP both the basic idea and experimental process of RIP are simple and straightforward, RIP has several unique features that are important to keep in mind in order to obtain meaningful results.

Several basic observations about RIP are worth recapitulating. First, in contrast to ChIP, the amount of input RNA required to give a suitable RT-PCR signal (in the logarithmic range) differs from transcript to transcript in the same RIP, because transcript levels can differ dramatically from gene to gene. This often
precludes the use of multiplex RT-PCR for detecting several different RNAs at the same time. Second, when ChIP is used for studying the interaction of proteins with a gene, association with the gene region before activation of the gene (where the protein is absent) and after activation of the gene (where it is present), is often examined. Such an experiment is not meaningful for RIP, because there is no RNA expressed from the gene under noninducing conditions.

Third, it has been repeatedly observed that, at least for non-sequence-specific RNA-binding (transcription-related) proteins, cross-linking is not always restricted to the portion of the transcript predicted by protein function. For example, transcription factors typically cross-link much more readily to the beginning than to the end of the transcript (Gilbert et al., 2004). This could, of course, indicate that transcription complexes are more abundant at the 5′-end of the gene, perhaps reflecting that a proportion of polymerases abort transcription during progression through the coding region (Kristjúhan and Svejstrup, 2004). However, an alternative explanation that nicely illustrates a fundamental and important difference between RIP and ChIP should be pointed out. Whereas DNA is likely to behave spatially more or less like a “linear” molecule, there is good reason to believe that RNA is spooled into a defined ribonucleoprotein (mRNP) structure during transcription (Huertas and Aguiler, 2003; Svejstrup, 2003). Such cotranscriptional RNA packing could potentially result in transcription factors apparently being cross-linked better to regions in the beginning of the transcript, because these regions (in contrast to the corresponding DNA regions) remain in close proximity to the transcribing polymerase and associated factors even as the transcription complex reaches the end of the gene. The 5′-end of the RNA molecule is thus in contact with the protein along the entire length of the gene, whereas sequences at the 3′-end of the RNA are obviously only in contact after they have been produced. Conversely, this packing of RNA also means that proteins known to specifically associate with the 5′-end of the transcript (such as CBP, which binds the cap of the finished transcript and exports the mRNA from the nucleus; Izaurralde et al., 1995; Gorlich et al., 1996) might also be expected to cross-link to regions in the 3′-end. Indeed, preliminary evidence for this has been obtained.

Finally, as in ChIP, it is important to use primers for a predicted “non-binding site” in control reactions. If mRNA-binding proteins are studied, these control primers might, for example, be designed to detect specific tRNAs or rRNAs. Likewise, if cotranscriptional association of proteins with pre-mRNA is studied, primers across an intron-exon junction can be used. Since splicing occurs cotranscriptionally (and introns are exclusively nuclear), it is then possible to study nucleus-specific RNA interactions (Gilbert et al., 2004). Obviously, considerations about the basic differences between genomic DNA and its different RNA products impact significantly on the potential uses of RIP for research.

Critical Parameters and Troubleshooting
Because RIP is designed to specifically detect RNA, it is obviously of pivotal importance to take special precautions to avoid RNA degradation throughout the procedure. Routine use of nuclease-free water, addition of RNasin to all buffers, and use of filtered pipet tips is recommended.

Cross-linking in vivo and choice of epitope To obtain trustworthy results with RIP, it is extremely important to carry out good control reactions. The most simple and powerful control is to use the same antibody-coupled beads (and not just “specific antibody” versus “preimmune serum” or “beads alone”) in two extracts that differ only in whether or not the target protein carries an epitope tag. This is because most RNA species are much more abundant than DNA and are present in both the nucleus and cytoplasm, so the risk of nonspecific cross-linking or nonspecific communoprecipitation is much higher. Varying the formaldehyde cross-linking times has an impact on the efficiency and specificity of the reaction, and it is strongly advised that an initial time-course experiment be performed for each new factor studied.

Sonication and DNase I treatment The formaldehyde cross-linked yeast cells are lysed by the use of a bead-beating procedure, which also has the effect of shearing nucleic acids. It is not required or desirable to perform extensive sonication of the extract for RIP, presumably because RNA is already substantially sheared by bead beating. Although, in the authors’ laboratory, four rounds of sonication are typically optimal for ChIP, only one
or two rounds of sonication are used for a typical RIP experiment; however, this should be empirically determined.

The sonicated extracts are then treated with DNase I to remove DNA. It is important, at least initially, to perform control experiments during the RNA-detection phase in which reverse transcriptase is left out of the PCR reactions, to ensure that only RNA is detected in the experiment. It is also a good idea to perform a control reaction in which the extract is treated with RNase, again to ensure that it is indeed RNA that is being detected.

**Immunoprecipitation**

The authors of this unit have observed that immunoprecipitations are more specific when performed by overnight incubation, using unconjugated antibodies directly in DNase I-treated extracts. Protein A–Sepharose beads are then added the next day, but only after a high-speed centrifugation of the antibody-incubated extract to remove material that may have precipitated during the incubation. The authors typically use only 1 to 2 µl antibody per immunoprecipitation reaction. Less material is precipitated in this way, but the signal-to-noise ratio is improved, which may be of particular importance for RIP.

**Reversal of cross-links and RNA storage and detection**

ChIP procedures typically include a very long incubation at 65°C to reverse formaldehyde cross-linking (UNIT 21.3). The authors of this unit have found that incubations as short as 0.5 to 1 hr are sufficient for reversal of RNA-protein interactions. After reversal of cross-links, RNA is precipitated. Problems of reproducibility with “stored” versus “fresh” RNA are sometimes encountered, but storing the RNA according to the procedure described in the Basic Protocol does help somewhat.

**RT-PCR**

There are several key parameters for achieving optimal amplification. For example, it is very important to have a quality repeat pipettor that can reproducibly dispense 2-µl samples. Pipetting inaccuracies at this stage will lead to greater well-to-well variability and poorer reproducibility among identical samples.

The PCR conditions tabulated in the Basic Protocol are generally appropriate for most situations. The annealing temperature may have to be adjusted if the melting temperature of the primers is substantially above or below 55°C. The number of cycles might also have to be adjusted in some cases if reactions are not in the linear range.

For a typical measurement, input RNA is tested along with immunoprecipitated samples. Different dilutions of both input and immunoprecipitated material are tested to ensure that the RT-PCR reactions are in the linear range. Because different RNAs are present in different copy numbers (in contrast to DNA), the dilutions needed to achieve reproducible and reliable results will differ between different RNA molecules. Typically, the input material needs to be diluted ~500-fold relative to immunoprecipitated material. Due to the variability in RNA copy numbers, simultaneous analysis of multiple RNAs in a single reaction is often not possible.

It is crucial to include a control reaction in which the RT-PCR step is performed without adding reverse transcriptase, to ensure that the final product is due to RNA rather than contaminating DNA.

**Genome-wide analysis**

ChIP-on-chip experiments have become a common feature of research reports over the last couple of years (see, e.g., Robert et al., 2004). Whereas RIP-on-chip has not yet been reported, there is no reason why such experiments cannot be performed (see UNIT 21.3 for details on ChIP-on-chip).

**Anticipated Results**

The described technique should be capable of identifying the interaction of a protein of interest with RNA, starting with ∼10⁷ yeast cells. Although the protocol was developed for the detection of RNA interactions of a pre-mRNA-interacting protein in the nucleus (Gilbert et al., 2004), the authors of this unit anticipate that it can be used for detecting RNA interactions (mRNA, tRNA, rRNA) of proteins in the cytoplasm, mitochondria, or nucleolus, among other locations. An example result is shown in Figure 27.4.1.

Since it is well-known that formaldehyde makes protein-protein cross-links, one cannot assume that a positive RIP result means that a given protein is actually bound to the RNA. A positive result means that the protein is “in the vicinity” of the RNA—for example, associated via other proteins in a multi-subunit complex. This is true for chromatin immunoprecipitation as well as for RNA immunoprecipitation.

**Time Considerations**

The Basic Protocol may be completed in 2 or 3 days. On the first day, cells are fixed with
formaldehyde and harvested (1 hr). Preparation of chromatin extracts (2 hr) and immunoprecipitations (primary antibody incubation, usually overnight) may be carried out on day 1, while incubation with protein A–coupled beads (~2 hr), washing and elution (1.5 hr), and reversal of cross-links (1–2 hr to overnight) can be done on day 2. On the final day, the RNA is purified (<4 hr), and reverse transcriptase PCR amplified (<3 hr for the PCR program), and analyzed by gel electrophoresis (<2 hr, including 1 hr of gel running time). Alternatively, the samples are analyzed by reverse transcriptase real-time PCR (<3 hr, including 2 hr for the PCR program).

**Literature Cited**


**Key References**

Gilbert et al., 2004. See above.

Describes the technique from which the Basic Protocol was adapted.

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