Immunoprecipitation of mRNA-protein complexes

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Immunoprecipitation of mRNA-protein complexes is a method that can be used to study RNA binding protein (RBP)–RNA interactions. In this protocol, an antibody targeting an RBP of interest is used to immunoprecipitate the RBP and any interacting molecules from a cell lysate. Reverse transcription followed by PCR is then used to identify individual mRNAs isolated with the RBP. This method focuses on examining an association between a specific RBP-mRNA complex, and it is best suited for a small scale screening of known or putative binding partners. It can also be used as a second, independent method to verify RBP-mRNA interactions discovered through more universal screening techniques. We describe the immunoprecipitation protocol in practical detail and discuss variations of the method as well as issues associated with it. The procedure takes three days to complete.

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INTRODUCTION

This protocol details the procedure for the immunoprecipitation (IP) of mRNA-protein complexes as shown in Zielinski *et al.*[1] It can be used for detecting mRNA-protein interactions, as well as to verify results obtained by the PAIR technique[2]. In the immunoprecipitation methodology, an RNA binding protein (RBP) is immunoprecipitated from a cell lysate, followed by reverse transcription (RT) of the immunoprecipitated material and PCR with primers targeting the interacting mRNA (**Fig. 1**). This method is based on the use of specific antibodies and specific PCR primers to examine the interaction within the targeted RBP-mRNA pair, and it is therefore best suited for a small-scale study of known or putative ribonucleoprotein interactions. Immunoprecipitation of ribonucleoprotein complexes has also been used in combination with cDNA arrays for a more universal screening of RBP-interacting mRNAs (ref. 3).

There are several key factors to consider when planning to use this methodology. The first is the choice of antibodies. The antibodies should be suitable for immunoprecipitation and have a low level of nonspecific binding. Any set of immunoprecipitation samples should include negative controls, such as an immunoprecipitation done with an unrelated antibody of the same type as the antibodies of interest and a "mock" immunoprecipitation done without any antibody to control for nonspecific binding to protein A–agarose beads. Another key factor for the success of this approach is the optimization of PCR conditions. The user should choose parameters according to the specifics of primer design (annealing temperature), product size (extension time) and target mRNA and protein abundances as well as the immunoprecipitation efficiency (number of cycles or PCR rounds needed). Too few PCR cycles will not yield detectable amounts of the PCR product; too many will saturate the PCR reaction and may result in nonspecific background bands. For best results, nested PCR should be performed; i.e., different primer pairs should be used for the two PCR rounds. Other factors that can be altered and that will influence the outcome are the starting material, i.e., the number and type of cells used for immunoprecipitations, and the lysis buffer. If a protein or mRNA is expressed at a low level or the interaction between the two is weak or rare, more starting material may be needed. Choice of lysis buffer may influence the solubility of the target protein and the ability to precipitate the intact protein-mRNA complex.

Experimental design

This protocol is based on rat cortical neurons, but any other cell type can be used according to user's preferences. The number of cells needed for a detectable signal in immunoprecipitation varies for each antigen and needs to be tested by the user. Other lysis buffers can be used depending on the target protein. This protocol uses protein A–agarose for separating the antibody-antigen complexes from the rest of the protein lysate, but protein G agarose might work better in some cases. Proteins A and G have different affinities for different antibody classes and for antibodies from different species. The user should determine which is optimal based on the types of antibodies being used for immunoprecipitation.

MATERIALS

REAGENTS

- Cells of choice
- Polysome lysis buffer (see REAGENT SETUP)
- Antibodies of choice
- Protein A–agarose (Invitrogen, cat. no. 15918-014)
- Phenol–chloroform–isoamyl alcohol mixture (Sigma-Aldrich, Fluka BioChemika Ultra, cat. no. 77618)
- Yeast tRNA (1 mg ml^{-1})
- Sodium acetate, 3 M
- Absolute ethanol
- Superscript III reverse transcriptase (Invitrogen, cat. no. 18080-093)
- AccuPrime Supermix I (Invitrogen, cat. no. 12342-010)
- Primers of choice (nested primers are recommended for the two rounds of PCR)

EQUIPMENT

- PCR thermal cycler

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REAGENT SETUP

Polysome lysis buffer 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 0.5% Nonidet P-40, 1 mM DTT, 100 U ml^{-1} RNasin RNase inhibitor (Promega, cat. no. N2511), 2 mM vanadyl ribonucleoside complexes solution (Sigma-Aldrich (Fluka BioChemika), cat. no. 94742), 25 μl ml^{-1} protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich, cat. no. P8340). Use polysome lysis buffer as described for lysing the cells; for washes it can be used without RNase and protease inhibitors.

Variations of the buffer (i) Polysome lysis buffer with 1 M urea; (ii) polysome lysis buffer with 0.1% SDS and proteinase K (30 μg per 100 μl).

PROCEDURE

Day 1: Lysate preparation ● TIMING 3+ h

1| Harvest rat cortical neurons in polysome lysis buffer. Use one 10-cm dish (4.8×10^6 cells) for each immunoprecipitation. Add 1 ml of polysome lysis buffer per 10-cm dish, scrape with a cell scraper to detach cells and transfer lysate to a tube on ice.

2| Centrifuge at 16,000*g* for 15 min in a microcentrifuge at 4 °C.

3| Pool the supernatant from all aliquots; transfer to a 15 ml tube.

4| Equilibrate 50% protein A–agarose slurry in polysome lysis buffer by washing twice with 0.5 ml of polysome lysis buffer and then restoring the original volume with lysis buffer. The amount of 50% agarose slurry needed is 50 μl per every 1 ml of lysate for each of the two preclearing steps and the immunoprecipitation step. For example, for every 4 ml of lysate, equilibrate three 200-μl aliquots of slurry. Use two of these aliquots for preclearing, and divide the remaining 200 μl into four 50 μl aliquots for the immunoprecipitation.

5| Preclearing: add one aliquot of equilibrated protein A–agarose beads to the lysate (50 μl of 50% agarose per 1 ml lysate) and incubate rotating at 4 °C for 1 h.

6| Divide lysate into microcentrifuge tubes and centrifuge briefly to collect beads. Pool supernatant and transfer to a new 15 ml tube.

7| Repeat Steps 5 and 6.

8| Divide lysate into 1 ml aliquots.

9| Add antibodies to the aliquots and incubate rotating at 4 °C overnight. Include antibodies against the target proteins, a negative control antibody of the same type as antibodies of interest and one aliquot of lysate without any antibody. This will be a negative control for nonspecific binding to protein A–agarose beads. Note: the amount of antibody needed varies for different antibodies and needs to be determined by the user. Also, not all antibodies are suitable for immunoprecipitation. Manufacturers often have information about suitability and recommended concentrations.

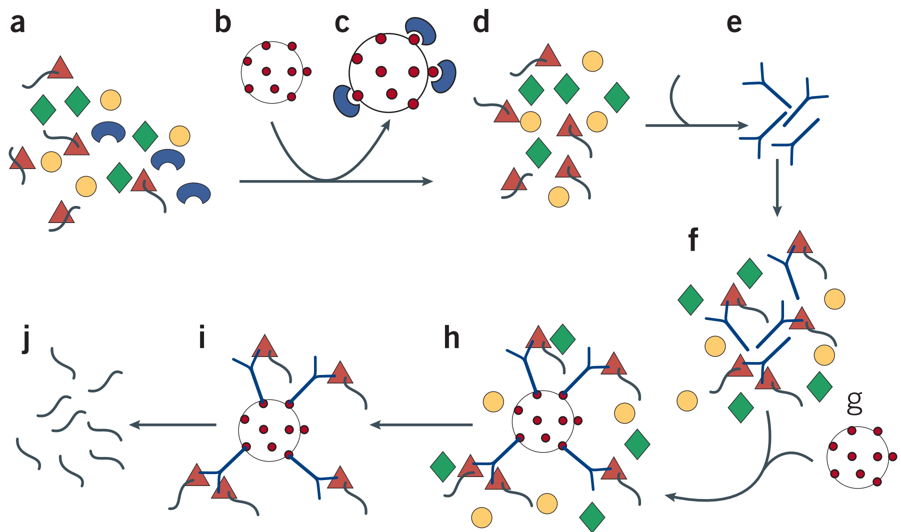

Figure 1 | (**a**–**c**) Protein lysate, **a**, is incubated with protein A–agarose beads, **b**, to preclear any nonspecific binding of the lysate to the beads, **c.** (**d**–**f**) The precleared lysate, **d**, and antibodies, **e**, are incubated together to allow the formation of antibody-antigen complexes, **f.** (**g**,**h**) Protein A–agarose beads, **g**, are then added to immunoprecipitate the antibody-antigen complexes, **h.** (**i**,**j**) Unbound proteins are washed off, **i**, and mRNA, **j**, is separated from the bead-antibody-RBP complexes for analysis by RT-PCR. Curved lines, RNA; red triangles, target RBP; green, yellow and blue shapes, lysate proteins; Y-shape, antibodies; white circles with red spots, Protein A-agarose.

Day 2: Immunoprecipitation ● TIMING 6.5+ h

10| Add 50 μl of protein A–agarose beads to each aliquot. Rotate at 4 °C for 4 h.

11| Centrifuge briefly to collect beads and discard the lysate.

12| Wash beads with 0.5 ml polysome lysis buffer four times. Rotate at 4 °C for 5 min for each wash.

13| Wash four times with polysome lysis buffer including 1 M urea, as above.

14| Resuspend beads in 100 μl of polysome lysis buffer with 0.1% SDS and 30 μg proteinase K. Incubate in a heating block at 50 °C for 30 min.

15| Add one volume (100 μl) of phenol–chloroform–isoamyl alcohol mixture and vortex to mix. Centrifuge for 1 min to separate phases. Recover (upper) water phase.

16| Repeat Steps 14 and 15 one or two times.

17| Add 10 μl yeast tRNA (1 mg ml^{-1}), 12 μl 3 M sodium acetate and 250 μl ethanol to 100 μl of water phase and mix. Ethanol-precipitate at –20 °C overnight. Note: other carriers such as linear acrylamide or glycogen can be used instead of yeast tRNA.
▲ **CRITICAL STEP** Cutting down the precipitation time may result in loss of material at this step and a negative PCR result.
■ **PAUSE POINT** Precipitations may be carried out for a longer time if needed.

Day 3: RT-PCR ● TIMING 8 h

18| Centrifuge for 20 min at 4 °C in an microcentrifuge at full speed (16,000*g*).

19| Remove ethanol thoroughly and let the pellet air-dry until all liquid has evaporated. Avoid air-drying for too long, as this will make resuspending the pellet difficult.

20| Resuspend in 13 μl RT mixture containing dNTPs (500 μM each) and 100 nM gene-specific primer. Reverse transcribe using Superscript III according to the manufacturer's instructions. Note: it is also possible to use an oligo-dT primer or random primers for RT. If using an oligo-dT primer for RT, the subsequent PCR primers should be targeted to the 3′ region of the mRNA, close to the poly-A tail.
■ **PAUSE POINT** Proceed directly to PCR or store RT reactions at –20 °C for later use.

21| Set up 20-μl PCR reactions using Accuprime Supermix I according to the manufacturer's instructions. Pipet 1 μl of the 20 μl RT reaction into a 20 μl PCR reaction for template. Run PCR for 20–30 cycles.

22| Set up another set of PCR reactions; use 1 μl of the first-round PCR reaction as a template for each 20 μl second-round PCR reaction. Run PCR for 20–35 cycles. Note: other PCR systems may be used. Different systems may give different results; sensitivity and amount of background may vary.

23| Visualize by running 10 μl of each of the 20-μl PCR reactions in an agarose gel.

● **TIMING**

This protocol can be carried out in 3 d, the first two of which need to be consecutive. The first day is a half-day consisting of the preparation of the cell lysate and preclearing steps. The second day is a full work day; however, the first half of it includes an incubation time of 4 h. At the end of the second day the stopping point is an ethanol precipitation of the immunoprecipitated RNA, which can be carried out for an extended period of time if needed. The third day consists of the

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RT-PCR and running of gels and allows the most flexibility in terms of timing. One should expect that PCR may have to be repeated for each round of immunoprecipitation experiments even after optimal conditions for the other steps have been established, which might add a few extra days to the total time.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

PROBLEM	POSSIBLE REASONS	SUGGESTIONS
No PCR products	Not enough RBP-mRNA complexes isolated	Increase the amount of cell lysate
	Interaction between RBP and mRNA disrupted during the procedure	Optimize lysis buffer Use fresh cell lysate (do not freeze) Use a less stringent buffer for washing the beads
	Antibody not immunoprecipitation compatible	Use a different antibody
	Antibody complex not binding to the beads	Choose the optimal beads to use—i.e., protein A vs. protein G—or use a different antibody
	PCR conditions not optimal	Optimize PCR parameters independently of the immunoprecipitation procedure Use PCR primers that bind close to the RT primer binding site Use nested PCR primers Increase the number of PCR cycles (up to 35 for each round of PCR)
PCR products in negative control samples	Nonspecific binding of cell lysate components to control antibodies or agarose beads	Increase the number, time and stringency of washes Use antibodies of high specificity
	Contamination of PCR reagents	Use new PCR reagents to eliminate potential contamination
	Genomic DNA contamination in immunoprecipitation samples	Treat the RNA sample with DNase before RT to eliminate the possibility of genomic DNA contamination

ANTICIPATED RESULTS

An optimal result would be PCR products of the expected size from the immunoprecipitations done with antibodies recognizing the target RBPs, whereas the negative controls (immunoprecipitations done with an unrelated antibody and no antibody) should not yield these PCR products.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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