

RNA-binding Protein Immunoprecipitation (RIP) to Examine AUF1 Binding to Senescence-Associated Secretory Phenotype (SASP) Factor mRNA

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[Abstract] Immunoprecipitation and subsequent isolation of nucleic acids allows for the investigation of protein:nucleic acid interactions. RNA-binding protein immunoprecipitation (RIP) is used for the analysis of protein interactions with mRNA. Combining RIP with quantitative real-time PCR (qRT-PCR) further enhances the RIP technique by allowing for the quantitative assessment of RNA-binding protein interactions with their target mRNAs, and how these interactions change in different cellular settings. Here, we describe the immunoprecipitation of the RNA-binding protein AUF1 with several different factors associated with the senescence-associated secretory phenotype (SASP) (Alspach and Stewart, 2013), specifically IL6 and IL8. This protocol was originally published in Alspach *et al.* (2014).

Materials and Reagents

A. Tissue culture

1. DMEM (Sigma-Aldrich, catalog number: D6429)
2. Fetal bovine serum (FBS) (Sigma-Aldrich, catalog number: F2442)
3. Medium M199 (Sigma-Aldrich, catalog number: M7528)
4. Penicillin/streptomycin (Sigma-Aldrich, catalog number: P4333)
5. BJ human foreskin fibroblasts
6. Bleomycin sulfate (Sigma-Aldrich, catalog number: B8416)
7. BJ culture media (see Recipes)

B. General materials

8. Bradford Protein Assay reagent (Bio-Rad Laboratories, catalog number: 500-0006)
9. Polyclonal anti-AUF1 antibody (EMD Millipore, catalog number: 07260MI)
10. Normal Rabbit IgG antibody (Cell Signaling Technology, catalog number: 2729)
11. Protein A Dynabeads (Life Technologies, catalog number: 10002D)
12. MCLB (mammalian cell lysis buffer) (see Recipes)
13. NT2 buffer (see Recipes)
14. Buffer A (see Recipes)
15. Buffer B (see Recipes)
16. Buffer C (see Recipes)

C. Immunoprecipitation reagents

17. Tris
18. EDTA
19. NP40
20. NaCl
21. Phosphate-buffered saline (PBS)
22. SDS
23. Deoxycholate
24. $MgCl_2$
25. NaH_2PO_4
26. RNase OUT (Life Technologies, catalog number: 10777-019)
27. Proteinase K (Sigma-Aldrich, catalog number: P6556)
28. RiboPure RNA isolation kit (Life Technologies, catalog number: AM1924)

D. qRT-PCR reagents

1. dNTPs
2. Hexanucleotide mix (Roche Diagnostics, catalog number: 11277081001)

3. SuperScript II Reverse Transcriptase (Life Technologies, catalog number: 18064-014)
4. Power SYBR GREEN PCR master mix (Life Technologies, catalog number: 4367659)

E. Primers

1. IL6 forward: 5'-ACA TCCTCGACGGCA TCTCA-3'
2. IL6 reverse: 5'-TCACCAGGCAAGTCTCCTCA-3'
3. IL8 forward: 5'-GCTCTGTGTGAAGGTGCAGT-3'
4. IL8 reverse: 5'-TGCACCCAGTTTTTCCTTGGG-3'

Equipment

A. Tissue Culture

1. 37 °C forced-air incubator maintained at 5% CO₂ and 5% O₂ (Thermo Fisher Scientific, Forma Series II water jacketed CO₂ incubator)
2. Laminar-flow biosafety cabinet (Labconco Purifier Class II Biosafety Cabinet)

B. Immunoprecipitation

1. Spectrophotometer capable of reading at 450 and 595 nm
2. Dyna-mag magnetic bead separator (Life Technologies, catalog number: 12321D)
3. Micro centrifuge
4. End-over-end tube rotator (Thermo Fisher Scientific, catalog number: 400110Q)

C. qRT-PCR

1. PCR machine (Bio-Rad Laboratories, model: C1000 thermo cycler)
2. Real-time PCR detection system (Bio-Rad Laboratories, model: CFX96 Real Time System)

D. General equipment

1. Tissue culture cell scrapers

2. Refrigerated micro centrifuge
3. Heat block capable of reaching 70 °C

Procedure

1. BJ human foreskin fibroblasts were cultured at 37 °C, 5% CO₂, 5% O₂ in BJ culture media. Stress-induced premature senescence (SIPS) was induced by treating the cells with 100 µg/ml bleomycin sulfate for 24 h. Bleomycin sulfate-containing media was replaced with fresh media following the 24 h treatment. Cells are senescent 3 days following treatment with bleomycin.
2. Collect cells by scraping in PBS on ice (for a 15 cm culture dish, 4 ml of PBS was used). 3.5 million cells are required for each immunoprecipitation (IP) (IgG control and specific antibody). Cell number was determined using a hemocytometer.
3. Pellet cells by centrifugation at 1,500 rpm for 5 min at 4 °C.
4. To lyse cells, resuspend the pellet in 1 ml MCLB and incubate for 20 min at 4 °C with end-over-end rotation.
5. Centrifuge cell lysates at maximum speed for 20 min at 4 °C to remove cellular debris. Remove 1 ml of cell lysate to a micro centrifuge tube.
6. Remove 100 µl of protein lysate from each and place in a new Eppendorf tube. This will be used as the input for each sample.
7. Quantification of protein concentration was performed using the Bradford assay following manufacturer's protocols.
8. Prepare the magnetic beads for immunoprecipitation.
 - a. Wash 100 µl beads per IP three times in 0.1 M NaH₂PO₄ (for example: If 2 AUF1 immunoprecipitations and 2 IgG immunoprecipitations are to be performed, you will require 200 µl of beads for the AUF1 IPs and 200 µl of beads for the IgG IPs). Washes are performed by resuspending the magnetic beads, and then separating them from solution using a magnetic micro centrifuge tube rack.

- b. Resuspend washed beads in 500 μ l 0.1 M NaH_2PO_4 and add 30 μ g anti-AUF1 or IgG control antibody per IP (for example: If you are performing 2 AUF1 IPs, resuspend your beads in 500 μ l of 0.1 M NaH_2PO_4 and add 60 μ g of anti-AUF1 antibody).
 - c. Incubate beads with antibodies at room temperature for at least 1 h with end-over-end rotation.
 - d. Wash antibody-conjugated beads 3 times in buffer A by resuspending beads in buffer A and pulling them down using a magnetic micro centrifuge tube rack. Leave beads in buffer A until ready for IP.
8. Pre-clear 3 mg of protein per IP by incubating with 20 μ l non-antibody conjugated beads. Bring up to 1 ml volume with MCLB. Incubate for 30 min at 4 $^{\circ}\text{C}$ with end-over-end rotation.
9. At the same time, resuspend antibody-conjugated beads in NT2 buffer. At this time you need to aliquot the beads for the individual IPs. For example, if you are preparing to do 2 AUF1 IPs, resuspend the beads in 200 μ l NT2 buffer and separate them in to 2 tubes of 100 μ l each. Incubate for 30 min at room temperature.
10. Immunoprecipitate overnight:
 - a. Remove the beads from the pre-cleared protein lysate using a magnetic micro centrifuge tube rack, keeping the protein lysate and discarding the beads.
 - b. Remove the antibody-conjugated beads from NT2 buffer using a magnetic micro centrifuge tube rack, keeping the beads and discarding the NT2 buffer.
 - c. Resuspend antibody-conjugated beads in pre-cleared protein lysate.
 - d. Place tubes on end-over-end rotator. Incubate at 4 $^{\circ}\text{C}$ overnight.
11. Wash the beads twice in each of the following buffers by resuspending the beads and then separating them from solution using a magnetic micro centrifuge tube rack
 - a. Buffer A
 - b. Buffer B
 - c. Buffer C
12. Incubate beads for 30 min at 55 $^{\circ}\text{C}$ in 100 μ l NT2 buffer supplemented with:

- a. 0.1% SDS
 - b. 80 U RNase OUT
 - c. 30 µg proteinase K
13. Extract the RNA from the IP's and input samples by adding 1 ml of Trizol (included in the RiboPure RNA isolation kit) to the mixture described in step 12. Incubate the solution for 5 min at room temperature. Proceed with the RNA isolation protocol provided in the RiboPure RNA isolation kit. Elute the RNA in 24 µl H₂O.
14. The eluted RNA can now be used to generate cDNA for qRT-PCR.
- a. cDNA was synthesized using hexanucleotide primers and following the manufacturer's protocol provided with Super Script II Reverse Transcriptase.
 - b. PCR was performed with Power SYBR Green master mix following the manufacturer's protocol. The annealing temperature used for the primers that are outlined in the Materials and Reagents section is 60 °C.

Representative data

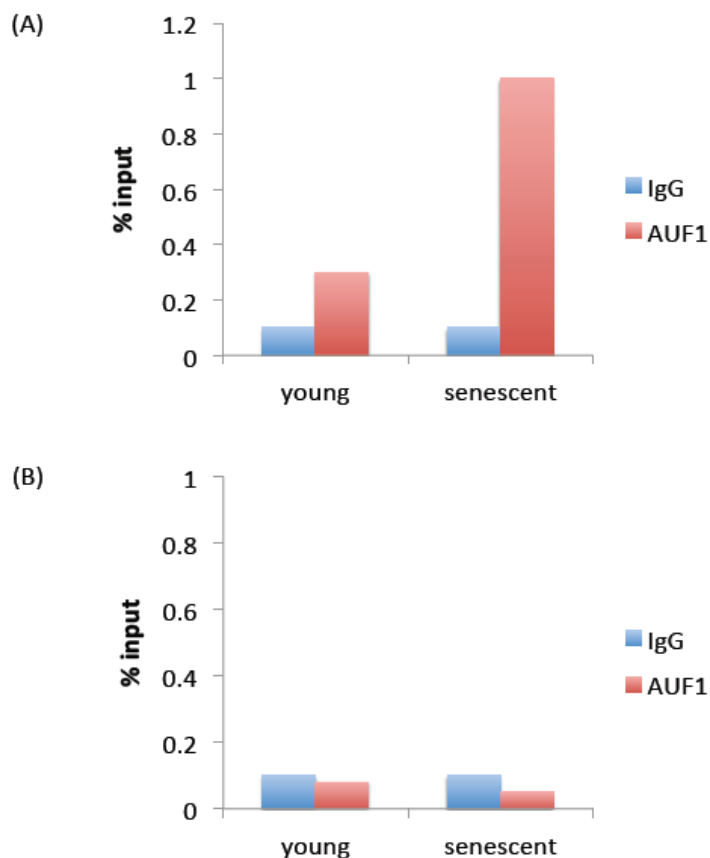


Figure 1. Representative results. Panel (A) represents expected results. The % input observed for AUF1 immunoprecipitation is above that observed for IgG immunoprecipitation, indicating that the AUF1 antibody pulled down more protein (and RNA) than the non-specific IgG antibody. Panel (B) represents a negative result, where immunoprecipitation with the AUF1 antibody did not pull down more protein (and RNA) than the non-specific IgG antibody.

Notes

1. Please see Figure 2 for a simplified experimental workflow.

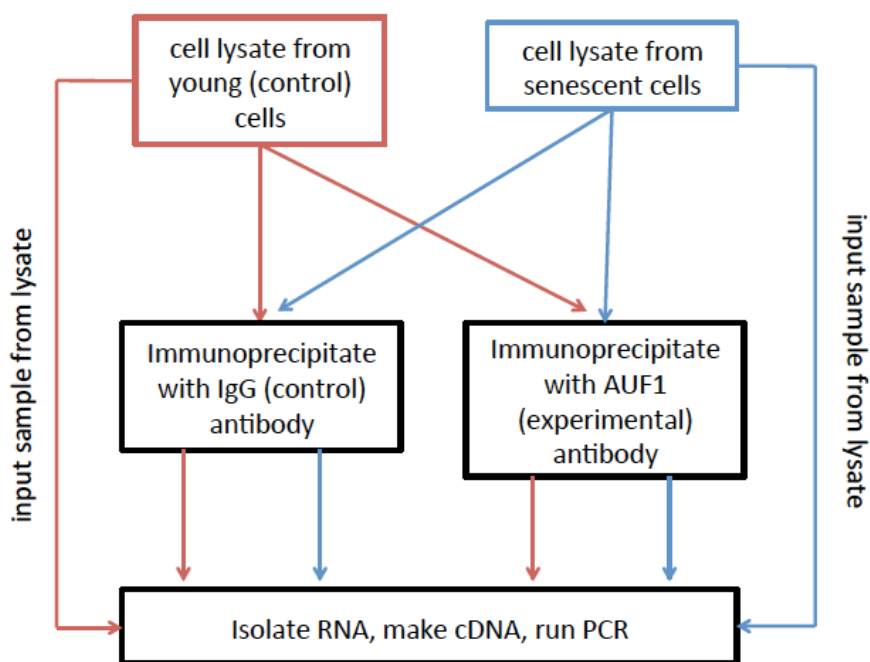


Figure 2. RNA Immunoprecipitation (RIP) workflow. Lysates from each treatment are divided into three groups: 1) Inputs, which are used to normalize to overall expression level of the RNAs of interest, 2) IgG immunoprecipitations, which are used to set the background level of immunoprecipitation, and 3) AUF1 immunoprecipitations, used to investigate the relative level of AUF1 binding to your RNA of interest.

Recipes

1. BJ culture media
 - DMEM
 - 15% heat-inactivated fetal bovine serum
 - 15% medium 199
 - 1% penicillin/streptomycin
2. MCLB (mammalian cell lysis buffer)
 - 50 mM Tris (pH 8.0)
 - 5 mM EDTA
 - 0.5% NP-40

- 100 mM NaCl
3. NT2 buffer
 - 50 mM Tris (pH 7.4)
 - 150 mM NaCl
 - 1 mM MgCl₂
 4. Buffer A
 - 1x PBS
 - 0.1% SDS
 - 0.3% deoxycholate
 - 0.3% NP-40
 5. Buffer B
 - 5x PBS
 - 0.1% SDS
 - 0.5% deoxycholate
 - 0.5% NP-40
 6. Buffer C
 - 50 mM Tris-HCl (pH 7.4)
 - 10 mM MgCl₂
 - 0.5% NP-40

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References

1. Alspach, E., Fu, Y. and Stewart, S. A. (2013). [Senescence and the pro-tumorigenic stroma](#). *Crit Rev Oncog* 18(6): 549-558.
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