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Effective identification of RNA-binding proteins using RNA Immunoprecipitation

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1 Works for me dx.doi.org/10.17504/protocols.io.bjpbkmin

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Chemicals Required 1d

1 1. Nuclear isolation buffer; 1.28M Sucrose, 40mM Tris-HCL (pH=7.5), 20mM $MgCl_2$, 4% Triton-X

For 100ml

1.28M sucrose	43.21g
1M Tris-HCL 7.5	4ml
100mM $MgCl_2$	20ml
100% Triton-X	4ml

2. R.I.P. buffer; 150mM KCl, 25mM Tris-HCL (pH=7.5), 5mM EDTA, 0.5mM DTT, 0.5% Noditet P-40

For 250ml

150mM KCl	2.795g
1M Tris-HCL 7.5	6.25ml

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50mM EDTA 8.0 2.5ml

1M DTT 125ul

100% NP-40 1.25ml

1x Protease inhibitor (add fresh every time)

100U/ml RNAase inhibitor (add fresh every time)

Autoclave and keep chilled at 4.

Cross linking and Lysis of The cells 6h

2 **Crosslinking is suggested to make stable interactions**

For cell lines

For each Ab to be tested, 1x100mm plates must be used.

1. Add 270ul of 37% Formaldehyde into 10ml culture media (P100)

2. Incubate at 37 for 15'

3. Add 1ml 1.25M Glycine and incubate 5' at RT

4. Wash cells with ice-cold PBS x2

5. Add 1ml of PBS (200ul), Nuclear isolation Buffer (200ul) and water (600ul)

6. Scrap cells and collect in a new tube

7. Centrifuge in 14,000rpm for 2' at 4 deg.

8. Remove supernatant

9. Add/resuspend with 1ml freshly made RIP Buffer (10ul protease inhibitor+100U/ml RNAase inhibitor)

10. Incubate on ice for 10'

11. Sonication : 15 sec on/45 sec off (x6 times for each sample)

12. Centrifuge at 14,000rpm for 15' at 4 deg.

13. Transfer supernatant into a new single tube (keep 10% input and 10ul for Western blot analyses of the RNA-Binding protein of interest-add 10ul 2x Loading buffer+DTT.Boil for 10' in 99 and perform Western Analysis)

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Immunoprecipitation of Crosslinked Protein/RNA Complexes 1d 6h

3

1. Precleaning : add 30ul of protein G bead for precleaning

2. Incubate for 1h at 4 deg. With rotation

3. Centrifuge at 3000rpm for 2' at 4 deg.

4. Transfer supernatant in a new tube

5. Add 1-5ug of antibody or IgG (negative control)

6. As positive control use anti-SNRNP70 Ab Millipore Cat. # CS203214

7. Incubate overnight at 4 deg. With rotation

8. Add 50ul Magnetic Beads and incubate with rotation 1-4 hours at 4 deg.

9. Spin beads at 3000rpm for 2’ at 4 deg.

10. Resuspend in 500ml RIP buffer.

11. Rotate at 4 deg. For 5 minutes each. Repeat x3 for a final of 4 washes.

12. Resuspend beads with 100ul RIP buffer+1ul RNAase inhibitor (do not forget 10% input)

13. Incubate at 70 deg. For 1h to de-crosslink samples

14. Spin down and remove supernatant.

RNA purification and Reverse Transcription 4h

4

RNA Purification

1. Resuspend beads in 100ul RIP buffer with 0.1% SDS and 30ug protease K. (For 1ml add 10ul from 10% SDS and 3ul from 10mg/ml protease K).

2. Incubate at a heating block in 55oC for 30’ with shaking.

3. Repeat step 1-2 two times. For the input samples do the step once.

4. Add one volume of phenol-chloroform-isoamyl alcohol mixture and vortex to mix. Centrifuge for 1 min to separate phases. Recover (upper) water phase (you can aspirate with no problem 175ul)

5. 10ul yeast tRNA (1mg/ml), 12ul 3M sodium acetate and 250ul ethanol to 100ul water phase and mix. Ethanol-precipitate at -80 overnight. Note : other carriers such as linear acrylamide or glycogen (5ul from 20ug/ul stock-I use Invitoegen Glycogen) can be used instead of yeast tRNA.

6. Centrifuge at 14,000rpm for 30 min at 4 deg. And discard the supernatant carefully.

7. Wash the pellet once with 80% ethanol. Centrifuge at 14,000rpm for 15 min at 4 deg.

8. Discard the supernatant carefully and air dry the pellets

9. Re-suspend in 20ul RNAase-free water and place tubes on ice.

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10. Typically, proceed to RNA quality control of the input with Nanodrop. Ideally we expect then 260/230 Ratio to be close to 2.00

Reverse Transcription

1. Label the appropriate number of PCR tubes 0.2ml for the number of samples to be Analysed and place on ice.

2. Set the reaction below.

Reagent	Volume/reaction (ul)
RNA	9.0
5x RT Buffer	4.0
20x Enzyme mix	1.0
RT Primer Mix	1.0
RNAse free-water	5.0
Final Volume	20.0

3. In a Thermo cycle set the following program

RT Reaction 37 60min

Stop the reaction 95 5 min

Hold 4 Hold

4. Remove the PCR Tubes. Dilute the reaction with 180ul Nuclease free water (10x dilution). Reactions can be stored at -20.

Positive Control RT-PCR

5

1. Label the appropriate number of 0.2ml PCR tubes for the number of samples to be analyzed and place on ice.

At a minimum, there will be 4 samples to undergo PCR using the RIP Primers : cDNA from positive (anti-SNRNP70) and negative control antibody (Normal Rabbit IgG) immunoprecipitations, input and a no template tube as a Control fro DNA contamination.

The RIP primers are specific for the human U1 snRNP gene. F : 5'-GGG AGA TAC CAT GAT CAC GAA GGT-3', R : 5'-CCA CAA ATT ATG CAG TCG AGT TTC CC-3'

2. Add 2ul of appropriate sample to the PCR tube and return on ice.

3. Add the following reagents

Reagent	Volume/reaction (ul)
DNA	2.0
10x PCR Buffer(-MgCl2)	10.0
MgCl2 (50mM)	0.6

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2.5mM dNTPs	1.6
RIP Primers U1 snRNA	0.8
Taq (5U/uL)	0.4

4. Set up the following PCR reaction

Initial Denaturation 94deg. 3’

Denature 94 deg. 20’’

Anneal 60 deg. 30’’ x20

Extension 72 deg. 30’’

Final Extension 72 deg. 2’

5. Run on 2% agarose gel. The expected size of the PCR Product is 100 base pairs. You should see bands in anti-SNRNP70 RIP and in 10% input.

RIP-qPCR/RIP-Seq

6 1. Add 2ul of the cDNA sample to the PCR plate suitable for the real time instrument of choice, performing in triplicates.

2. Prepare the following master mix.

Reagent	Volume/reaction (ul)
cDNA	2
SYBR-Green Master Mix	12.5
RT Primer Mix	1.0
RNAse free-water	9.5
Final Volume	23.0

3. Run on this program

Initial Denaturation 95deg. 10’

Denature 95 deg. 15’’

Anneal and extension. Tm-5 1’ x40

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