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Effective Identification of Protein-Protein Interaction using RIME-IP

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Buffer Preparation 2h

1 This is a modification of the Mohammed et al., 2016 (Nature protocols, 11(2), p.316.), that it has been edited and optimized for the need of Tsichlis Lab. The authors declare no conflicts of interest.

Immunoprecipitation 2d

2 Prepare the following buffers (**usually prepare 25ml of each, store at 4 °C And make fresh every month**) at the final concentration listed :

•1M Tris pH=7.5

for 100mL add 12.114g base
Adjust pH to 7.5

•1M NaCl
For 100mL add 5.844g

•50mM EDTA
For 100mL add 1.4612g

•5mM EGTA
For 100mL add 0.19g

•50mM DTT
For 1,5mL add 75uL from 1M stock

Lysis Buffer 1 (LB1)-will contain Cytoplasmic Proteins

For 25ml Volume
•50mM Tris-HCL ph 7.5 - add 1.25mL from 1M stock
•20mM NaCl- add 500uL from 1M stock
•1mM EDTA - add 500uL from 50mM stock
•0.5% NP-40 - add 125uL from stock (Sigma # 74385-1L)
•0.25% Triton X-100 - add 62.5uL from Stock
•10% Glycerol - add 2.5 ml from stock
•1mM DTT - add 75uL from 50mM stock

Lysis Buffer 2 (LB2)- wash to remove LB1 and cytosolic protein

For 25ml volume
•10mM Tris-HCL ph 7.5 - add 250uL from 1M stock
•20mM NaCl- add 500uL from 1M stock
•1mM EDTA - add 500uL from 50mM stock
•0.5mM EGTA - add 500uL from 5mM stock
•1mM DTT - add 75mL from 50mM stock

Lysis Buffer 3 (LB3)

•10mM Tris-HCL ph 7.5 - add 250uL from 1M stock
•100mM NaCl- add 2.5ml from 1M stock
•1mM EDTA - add 500uL from 50mM stock
•0.5mM EGTA - add 500uL from 5mM stock
•0.1% (w/v) sodium deoxycholate
•0.5% (v/v) N-laurylsarcosine - add 625uL from 20% Sigma stock
•1x protease inhibitors-add 250uL from 100x stocks

3 Day 1

Antibody-Bead Conjugation Day One

1. Magnetic Beads (Pierce Cat No. # 88802), lightly vortex to resuspend. WIth a cut pipette tip, transfer 50-75uL (some will be lost during the washes) of beads to 1mL Eppendorf tube. Place magnetic stand and remove supernatant.
2. Add 500uL ice-cold PBS and lightly vortex to resuspend. Spin briefly with table-top microcentrifuge at 2000xg for 1'. (Tip : by vortexing in higher speed you are destroying the magnetic beads). Place tube in magnetic stand and remove PBS wash. Repeat 2x for a total of 3 washes.
3. Add 300uL of LB3 to beads. Repeat vortex, spin and wash removal as in step 2 for a total of 3 washes.

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4. Add antibody for IP (according to company specifications-5ug) to top of the beads and incubate on ice for 10 min. Add 300uL LB3 to beads. Vortex tube lightly to resuspend beads. Rotate bead/antibody mixture at **4 °C** O/N.

4 Day 2

Harvest cells

1.Add media from 15cm plate at 80-90% confluent (around $2x10^7$ cells) to 50mL Falcon. Wash the cells with 5mL-ice Cold PBS and add to Falcon. Add 3mL ice-cold PBS and collect cells with scraper. Add to Falcon. Rinse plate with 5-10mL ice-cold PBS and add to Falcon.
2.Pellet cells by centrifugation at 400xg for 6 min. Aspirate media
3.Resuspend cell pellet in 1mL of ice-cold PBS and transfer to Eppendorf tube. Rinse Falcon with 500uL of ice-cold PBS and transfer to Eppendorf. Spin as in step 2.
4.Remove PBS. Wash cell pellet with 500uL PBS, spin as before and remove PBS wash.
5. Resuspend cell pellet in 750uL of LB1+freshcocktail inhibitor . Rotate the tube at **4 °C** for 10 min. Centrifuge at 14,000rpm at **4 °C** for 6 min. Remove supernatant (cytosolic proteins) and label tube if needed for further analysis.
6. Add 500uL LB2+inhibitors to top of nuclei. Centrifuge at 12,000rpm **4 °C** for 6 min. Remove supernatant
7. Resuspend nuclei in 300uL LB3+inhibitors. Sonicate cells on high 30s on/off for 5 min. Centrifuge to pellet debris max. Centrifuge to pellet debris max speed at **4 °C** for 10 min. Do not forget the 10% input.

Antibody-Beads Conjugates washes

1. Place beads on magnetic rack and remove supernatant. You can save supernatant and run in gel long with your samples to check to saturation of beads-Ab.
2. Wash beads with 300uL of LB3, vortex lightly to resuspend, spin briefly and remove supernatant. Repeat wash 4x for a total of 5 washes.
3. Quantitate amount of protein with Bradford prior to IP to ensure equal loading. Add the appropriate LB3 protein volume to antibody-bead conjugate. Rotate **4 °C** O/N

Western Blot analysis 2d

5 Day 3

1. Place beads in magnetic stand and remove protein mixture. Add 500uL LB3 to beads to wash, lightly vortex, spin briefly, place in stand and remove wash. Repeat this step at least 4x for a total of 5 washes.
2. For WB analysis, add about 50uL 5x Sample Buffer and 2.5uL 50mM DTT. Boil in **99 °C** for 10'. Place on magnetic rack and remove supernatant. This is your sample for Western Blot analysis.

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