PIC Cross-linking and Immune Precipitation

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Reference: Fishburn et. al., 2005, Molecular Cell, vol. 18 #3: Experimental Procedures pg. 376

Immobilized Template assay (Hahn lab website)

Notes:

- DTT must be omitted from all buffers
- 1 M KOAc seems to be the upper limit for solubility of NP40. If a precipitate forms, lower the KOAc concentration slightly.
- A 'no UV' control is not required for this procedure (see step 7).

Procedure

1. Prepare templates per standard protocol
2. Dialyze extracts using 0.02 micron discs on buffer C + 75 mM ammonium sulfate + protease inhibitors for 1 hr at 4 deg to remove DTT (100-150 microliter extract per 10ml buffer C)
3. Add PEAS labeled activator to templates and bind 10' at RT
4. Make reaction mix and form 2X PICs (200 microliter) per standard protocol
5. Wash PICs 3 x 400 microliter txn wash buffer
6. Resuspend PICs in 200 microliter txn wash buffer
7. Do not split reactions into 100 microliter aliquots as is standard for cross-linking reactions
8. Cross-link samples in the UV oven: energy = 1250 units with 365nm bulbs
9. Concentrate beads, discard supernatant
10. Resuspend in 20 microliter txn wash buffer + 2 microliter 1 M DTT
11. Incubate 10' at RT, mix occasionally
12. Concentrate beads, discard supernatant
13. Resuspend beads in 100 microliter disruption buffer (txn wash buffer + 1 M KOAc final concentration)
14. Incubate 30' at RT with gentle mixing (Dynal mixer) to disrupt PICs
15. Concentrate beads, transfer supernatant to new tube containing 10 microliter M2-±Flag Sepharose
16. Incubate IP reactions 1 hr at RT with mixing on Dynal mixer
17. Collect Sepharose resin by centrifugation, discard supernatant
18. Wash resin 1 x 100 microliter disruption buffer
19. Wash resin 1 x 100 microliter txn wash buffer to reduce salt

20. Elute bound proteins with 15 microliter 1X SDS sample buffer + 1.5 microliter 1 M DTT: incubate at 70deg for 10' with mixing in Thermomixer
21. Transfer eluates to new tubes, store at -20 deg

Analysis

Analyze by Phosphorimager and Western:

1. Run samples on 4-12% Bis-Tris or other NuPAGE
2. Transfer to Immobilon FL membrane
3. Air dry membrane
4. Expose to Phosphorimager screen for 2-7 days
5. Rewet membrane in blocking buffer-PBS
6. Perform Western for ±Flag and/or other proteins of interest per standard protocol