Calcium Phosphate Transfection of 293T Cells

Required Solutions and Reagents

Lysis Buffer

50 mM Tris-HCl (pH 7.4)
1% Triton X-100
0.1% SDS
150 mM NaCl
1 mM EDTA
1 mM DTT (stock is 1 M) *
1 mM PMSF (stock is 100 mM in isopropanol) *
1 µg/ml Aprotinin (stock is 10 mg/ml) *
10 µg/ml Pepstatin (stock is 1 mg/ml) *
1 µg/ml Leupeptin (stock is 10 mg/ml) *
Aliquot and store at -20ºC.
*Add right before use.

Laemmli Buffer 4x

4.4 ml 0.5 M Tris-HCl pH 6.8
4.4 ml glycerol
2.2 ml 20% SDS
0.65 ml 1% bromophenol blue
0.5 ml beta-mercaptoethanol
Store at -20ºC.

Pre-blocked Protein-A or protein-G sepharose

1. Transfer 3-4 ml of protein A/G beads to a 15-ml conical tube. Quick-spin in a desktop centrifuge at 1000 rpm, aspirate the preservative.
2. Resuspend the beads in PBS. Spin again.
3. Repeat the wash 2 times.
4. Resuspend beads into 10 ml of 5% BSA.
5. Keep on ice for 30 minutes.
6. Wash the beads 3 times with PBS, resuspend the beads into equal volume of PBS, add sodium azide to 0.02% and store at 4^{o}C.

2X HBS

	For 1 l:
50 mM HEPES, pH 7.05	11.92 g
10 mM KCl	745.60 mg
12 mM dextrose	2.16 g
280 mM NaCl	16.36 g
1.5 mM Na_2PO_4	10 ml from 100x: 40.2 g $Na_2PO_4.7xH_2O$/l

Dissolve in 950 ml H_2O. Adjust pH to exactly 7.05 with 1 N HCl, bring up to 1 l with ddH_2O. Filter using .22 micron filter. Aliquot in 50 ml Falcon tubes. Store at -20ºC.

2M $CaCl_2$

Filter sterilize. Store in 1 ml aliquots at -20ºC. Stable at RT.
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Transfection

1. Twenty-four hours prior to transfection, inoculate 1 to 2 x10^6 cells/10 cm plate (or, inoculate 3x10^6 cells/plate 16-hours prior to transfection) . in 10 ml DMEM media + 10% BCS supplemented with L-glutamine and Pen/Strep.

   At the time of transfection, cells should be about 30-40% confluent.
2. The following day (20-24 hours later), transfect cells. Up to 15 to 20 µg DNA can be used for one 10-cm plate. If more than one plasmid is used for transfecting the same plate, equalize DNA amounts among different plates by adding vector plasmid DNA to some plates, so that the total amount of plasmids used for each plate is the same. For transfection:
   a. Prepare the following mix for 2 plates, in the same order :
      856 µl H_20
      124 µl 2 M $CaCl_2$
      15-20 µg DNA
      Vortex to mix.

   b. Slowly (drop-wise) add 1 ml of 2x HBS while aerating the mix using a 2 ml pipet.
   c. Drop 1 ml of the final mix around the plate, then mix gently. Place the plate back in the incubator.
3. Twelve to 16 hours after transfection, gently aspirate the medium and add 10 ml of pre-warmed fresh medium to the plates. Try not to disturb the fine DNA-$CaPO_4$ precipitates on the bottom of the plate.
4. The following day, harvest the cells and extract protein as described below.

Co-immunoprecipitation from Tx 293T cells

1. Harvest the cells 24-30 hours post-transfection. Remove media. Using a 10 ml pipet, flush the loosely attached 293T cells off the culture dish with ice-cold PBS. Transfer the cells to a 15-ml Falcon tube. Keep the tubes on ice.
2. Collect the cells by centrifugation (1000 rpm, 5 min). Aspirate PBS.
3. Lyse the cells by resuspending the pellets into 0.5 m cold lysis buffer. Using 2 ml pipet, gently resuspend the cells by pipetting up and down 3-5 times. Transfer to a 1.5-ml Eppendorf tube. Vortex for 3-5 seconds and keep on ice.
4. Add 25 µl 5 M NaCl to each tube and vortex. This step raises the [Na] to 400 mM for efficient extraction of nuclear proteins. Keep on ice for 5 min. Note: For low salt conditions skip this step and keep on ice for 20 min.
5. Add 0.5 ml cold ddH_2O. Mix well while adding H_2O. Microcentrifuge at full-speed for 10 min in at 4ºC.
6. Carefully transfer the supernatant to a new Eppendorf tube. It is critical not to transfer any insoluble particles to the new tube. Any insoluble protein transferred to the new tube will contaminate the IP pellet. Make sure to take same volume for each sample (e.g. 800 µl). Also avoid taking along the film on top.
7. Save an aliquot of the supernatant for analysis of protein expression (lysate input western, typically, use 1-10% of input next to IP).

8. Pre-clearing: Add 50 µl pre-blocked protein A or G Sepharose beads. Re-seal the cap and let rotate for ½ hour at 4ºC. Then spin in microcentrifuge at 4ºC for 5 min at 4000 RPM and remove and save supernatant.

   Determine empirically how much antibody to use in IP. Polyclonal antiserum usually works better than monoclonal anti-tag antibodies, which recognize a single epitope. Usually, much more antibody is need in IP than western blot. For a start, try 5-10 µg of purified antibody or 5-10 µl antiserum for each IP.
9. Add antibody to the supernatant. Rotate the tubes at 4ºC for 5 hours.
10. Add 60 µl (50% slurry) pre-blocked protein-A or G Sepharose beads to each tube. Re-seal the cap and let it rotate for another hour at 4ºC. (Alternatively, protein-A/G beads can be added together with antibodies in step 9).
11. Spin the tubes in a microcentrifuge at 4ºC for 5 seconds at 4000 rpm to collect the beads. After centrifuge stops, immediately turn the tubes (still sitting in their slots) 180 degrees, so the pellets are on the bottom side of the rotor). This will prevent the pellets from sliding loose while washing the other tubes.
12. Carefully remove the supernatant with 1 ml pipetman. Try not to remove any beads (you may leave 50 µl supernatant behind). Add 1 ml cold lysis buffer (minus protease inhibitors). Invert and tap the tube to loosen the beads.
13. Spin again as in step 11.
14. Repeat washing for total of 4 times. Run the last centrifuge at full speed for 10 seconds. Remove as much wash buffer as possible.
15. Add 45-60 µl Laemmli loading buffer to each pellet. Resuspend and boil for 5 minutes.
16. Spin to collect the beads to the bottom. Use 10-15 µl of supernatant per lane in a western blot.