Ni2+ pulldown under denaturing conditions (Jentsch lab)

Protocol is based on Sacher, Pfander and Jentsch 2005, *Methods in Enzymology* 399, pp392; modified by Sigurd Braun 11-06-07

General note on purifying ubiquitylated or SUMOylated conjugates: due to the high activity of de-conjugating enzymes (especially de-SUMOylases like Ulp1 and Ulp2), the protease inhibitor NEM might be added to the culture medium 30 min prior harvest (2.5 – 10 mM f.c. ; stock: 400 mM in EtOH, e.g. 2.5 g/50 mL); NEM is highly toxic, therefore it is recommended to quench the remaining supernatant after harvesting the culture with an equimolar amounts of DTT.

Cell lysis

- Harvest cell culture corresponding to 200 (400) OD; wash 1x in H20 and transfer to 15 mL conicals; store at -80ºC
- Resuspend the cell pellet in 6 (12) ml 1.85 M NaOH containing 7.5% b-mercapto-ethanol on ice for 20 minutes.
- Precipitate proteins by adding 6 (12) ml 55% TCA and incubate on ice for another 20 minutes.
- Spin at 4500x g for 15 minutes (e.g. Beckman, JS-5.3 rotor) and discard supernatant.
- Wash protein pellet 2x with -20 deg 5 mL cold acetone and spin briefly again.
- Solubilize protein pellet by adding 12 mL buffer A containing 0.05% Tween20 and nutating by room temperature for at least 1 hr.
  (note: solubilization might be facilitated by transferring the lysed material into 50 mL conicals prior spinning; after adding buffer A to the TCA-precipitated protein pellet, vortex briefly until the pellet becomes loose, then incubate on nutator and briefly vortex every 5-10 min the first 30 min; as an alternative, you may solubilize the pellet mechanically using a glass rod, followed by pipetting up and down the solution with a 10 or 25 ml glass pipette prior vortexing)
- Transfer the suspension into oak-ridge tubes and precipitate non-solubilized particles by spinning at 15,000x g for 20 minutes (Beckman, JA 25.50 rotor)
- Transfer supernatant into fresh 15 mL conical
- Optional: adjusting the protein amount of the solubilized material after measuring the protein concentration with BCA (Pierce) (e.g. 2-5 uL sample in 1 mL BCA)

Ni pulldown

- Add imidazol to a final concentration of 10 mM (stock solution 100mM: 0.34 g in 50mL buffer A; freshly prepared; depending on the background binding, 20 mM may also be used)

- Add 100 ul NiNTA magnetic beads (washed 3x in buffer A) and incubate on nutator at 4ºC over night;
  note: less NiNTA beads (i.e. 30-50uL) might be sufficient as well, depending on the amount of the input material and the abundance of the His-tagged protein
- Spin down briefly beads in clinical centrifuge (1500 rpm, 5 min) and take off the supernatant leaving 500-1000 uL. Resuspend and transfer to 1.5 mL tubes.
- Wash bound material using a magnetic eppendorf tube stand:
  3x with buffer A containing 0.05% Tween20
  5x with buffer C containing 0.05% Tween20
  depending on the background signal, 1-2 mM imidazole might be added either to both or to buffer A only (I use 1 mM in buffer A)
- Elute bound material with 30 ul 1% SDS by incubating at 65ºC for 10 minutes.
- Transfer eluate into fresh eppendorf tube, dry in speed vac and resuspend the eluated material in 15 ul H_2O to which 15 ul HU buffer is subsequently added.
- Denature protein material at 65ºC (not at 95º !) for 10 minutes prior loading to SDS gel.

Materials:

buffer A: 500 mL	6 M guanidinium chloride:	286.6 g
	100 mM NaH_2PO_4 :	6.0 g
	10 mM Tris pH 8.0:	0.6 g
buffer C: 500 mL	8 M urea:	240.2 g
	100 mM NaH_2PO_4:	6.0 g
	10 mM Tris pH 5.9	0.6 g
or 100 mL	8 M urea:	48.0 g
	100 mM NaH_2PO_4:	1.2 g
	10 mM Tris pH 5.9	0.12 g

note: due to dissociation of urea, check and adjust the pH of buffer C directly prior use (or rather prepare it fresh each time)

HU buffer: 8 M urea, 5% SDS, 100 mM DTT (add freshly!), 60 mM Tris/HCl pH 6.8 or 60 mM phosphate buffer, 1% brome phenol blue, 20% glycerol

Ni-NTA Magnetic Agarose Beads (Qiagen, #36111) or **Talon Magnetic Beads** (Clontech, #635636); Talon beads are supposed to have less background binding and are slightly less expensive