3½ -4hr

From Nurse pombe handbook

5.6 Immunoprecipitations

Breaking the cells

1. Collect 25 ODs of cells (by filtering or centrifugation).

2. Wash once with 10ml stop buffer

3. Resuspend in 1ml HB buffer and transfer to 2ml eppis. Spin down the cells and remove the supernatant. Cell pellets can be frozen at that stage. -70°C

4. add 100ul HB and 500ul glass beads

5. vortex with cell braker in CR for 5 min. Check under microscope (more than 60% should be broken) +5min

6. add 200ul HB, make hole with needle at the bottom of the eppendorf, place in clean eppendorf and spin for 1-2 sec to transfer liquid to new epp. - 6ml tube → epp 1.5ml. ~~10sec~~ 30sec.

7. spin 13k 15min. Transfer sup to new tube.

WE Keep 20ul extract+10ul HB + 30ul 2xsample buffer (300µl) to load on gel. what is Sample buffer Laemlli?

Keep 5ul+5ul 2xsample buffer w/o dyes for protein determination.

Protein concentration should be around 7-10ug/ul

Preparing beads

for protein A tagged proteins:

use 20ul human IgG agarose bead suspension/IP.

wash 3x 1ml HB. resuspend in 100ul HB/IP DO NOT VORTEX

~~For anti HA: prebind antibody to beads, use 3ul 16B antibody (1/100 dilution) and 20ul protein G sepharose beads per IP~~

~~wash protein G beads 3x 1ml HB, leave 500ul, add antibody, leave rotating for 30min at RT, wash 3x 1ml HB, resuspend in 100ul/IP~~

~~(check for complete binding to protein G, higher salt might be better)~~

Prepare beads for n+1 samples (eg use 100ul bead suspension for 4 Ips).

IPs

mix 200ul extract with 100ul bead suspension

rotate 2hrs in CR

spin 4k 20-30sec. keep sup (30ul+30ul 2xsample buffer for gel) SUP

wash 3x with 1ml HB (careful not to loose beads).

leave around 30ul, add 30ul 2xsample buffer (pellet would be 10x concent.)

boil all samples 5min 100oC Spin down beads + remove supernatant to new tube

Notes

- If more concentrated IP required, leave around 25ul and add 6ul 5xSB (around 20x)

-If IgGs might be a problem for western, use sample buffer w/o DTT or b-mercaptoethanol (will only work when antibodies are coupled to beads)

-To estimate IP efficiency, dilute IP sample after boiling (eg 5ul in 45ul 1xSB for equal loading to total)

-If background is a problem, try: adding salt to HB (150-500mM KCl), decrease the amount of beads added, preclear extract eg with protein A agarose, precoat the beads eg with BSA

Buffers

Stop Buffer

150mM NaCl, 50mM NaF, 10mM EDTA, 1mM NaN3. Prepare fresh before use. 10ml/sample

HB Buffer

25mM MOPS pH7.2, 15mM MgCl2, 15mM EGTA, 1mM DTT, 1% Triton-X100

phosphatase inhibitors: 60mM b-glycerophosphate, 15mM p-nitrophenylphosphate, 0.1mM sodium vanadate

proteinase inhibitors*: 1mM PMSF, 20ug/ml leupeptin, 40ug/ml aprotinin
* use Roche complete mini protease inhibitors instead one tablet per 7ml buffer

Note: proteinase inhibitor and EGTA concentrations are about 10x more than what normally used in other organisms, but S. pombe has high levels of proteinases, eg in nitrogen starved cells.

2x conc
laemli ?