PROTEIN EXTRACTION & 2D GEL

1. Grow cells in 100mL of SD+Glc (o/n) (+aa if they are needed).
2. Add 20ml of the previous step and growth cells in 3L of SD+Glc (overnight). OD_{600}=0.6-1.0

Prepare complete YEB buffer (add YEB + DTT, PMSF+Tablets Prot inh)
Prepare N_2 liquid.
Clean buckets with dH_2O.

3. Centrifugation at 4000rpm, 4ºC, 10min. (SALA B3065). Cells are transfered with a spoon to Falcon 50ml. (to ice) **Previously weight**.
4. Wash with 12mL H2Omq. Centrifugation at 4500rpm 3' x2.
5. Two washes with YEB buffer.**The first wash** with 10ml, **the second one** with final Vol 5mL (Cells + YEB).
   (1st DAY)
6. Liquid N2. Cells with syringe to form balls. Harvest cells with colander **Weight the cells**.
7. Keep in -80ºC (write: strain, Tº, Vol, date).

Prepare: Protein solution (for extraction) and Equilibration Solution (for isolectrofocusing)
9 criotubes and 5 falcon tubes of 15ml

(2nd DAY)

Clean everything with ethanol and change gloves to remove keratine presence
Spatula: with spoon (colect the pounder) & normal (remove ice and cells of the ball)

8. Griding (Retsch – mortar grinder).
   a. 10min cooling (N2 liquid – Second and a half line – refill constantly) 5-10min
   b. 10min 0 level.
   c. 5min 2 level.
   d. 5min 4 level
   e. 10min 8 level

Centrifuge at 4ºC
Surround with tissues the mortar grinder to dry

9. **(In the cold room)**Dissolve in 10mL Protein Solubilization Solution (lysis buffer). (5g of pellet wet weigth – 10mL of protein solution)
   a) Wait N_2 evaporation.
   b) Recover the pounder in a beaker (on ice) and add 10ml of Protein Solution (add a new tablet EDTA). Resuspend in cold room 10min.
   c) Protein extraction in falcon tube 50mL, centrifuge 4500rpm 15min 4ºC.
10. Supernadant is centrifuged at 4500rpm 10min 4ºC.
11. Distribute in criotubes the supernadant (1.5ml per tube).
12. Keep in -80ºC.
13. Thaw one criotube and apply step 14.
14. Apply RNase (Roche) (disolve in RNase buffer – 25mg stock + 500ul RNase buffer) <8ul RNase – 1.5 ml Prot extract > at RT 5min
15. Apply DNase (5mg/ml) <6ul – 1.5ml Prot extract> at 37ºC 5min.

Centrifuge at 4ºC.

16. If we want to increase the concentration add 6ml of Acetone (keep in -20ºC) (Falcon tube 10mL) + 1.5ml Prot extract. 15min on ice.
17. Centrifugation at 4000rpm 15min 4ºC.
18. Discard all supernadant and dry air (flux cabine) 5-10min.
19. Resuspend with Prot Solution(add 50mg DTT) (less than before i.e 1ml – 450ul the last time).
20. Columns (Optional – If you're interested in a protein fraction).
21. Bradford Protocol.docx.

Centrifuge at 4ºC
Cubetes are cleaned with washing-up liquid.

22. Prepare a dilution to obtain 0.5mg/ml in a final volume of 125ul (with protein solution + a little bit of DTT). Do per duplicate so prepare 250ul.
23. Spin 1min at 22000g 4ºC.
24. Take supernadant and put it on a new eppendorf.
25. Add 3ul of Bromafenol blue to each 125ul spin it and add the volume in one well.
26. Put the Strip 7cm pH 3-10NL face down. Passive rehydration 1h.

Preparar DTT 1M (500ul: 77mg of DTT)

27. IPG slips. Cover with mineral oil (1.5ml). Active rehydration (o/n).
28. IEF (6h)
    a. Put wicks with a little bit of DTT (to wet the wick, 5-10ul) over the electrodes (to avoid salt precipitation).
    b. Isoelectrofocusing step. (Select the number of strips in the program).

Clean bench & Materials with ethanol.

29. Prepare fresh 10% APS (1ml) before gel preparation.
30. Depending of your strip (7cm – small gel, 17cm – big gel).
    a. Add Running gel with Pasteur pipette (avoid bubbles). Limit: green border.
    b. Add a little bit of Isopropanol to remove bubbles (wait 15min to solidification). To follow the process we can see the rest of solution in Pasteur pipette.
    c. Remove isopropanol using a paper (tilt the cubete and decant).
    d. Add a little bit of H_2Omq (tilt several times to clean) and remove using a paper.
    e. Add Staking gel to overflow.
    f. Add the cone (avoid bubbles) (wait 15min to solidification).

Prepara EQ1 & EQ2

31. Using another well. Dry strip with paper and put it on well and wash with 2.5ml EQ1 (**EB 10ml + 200mg DTT**) 10min with shaking 50rpm.
32. Using another well. Dry strip with paper and put it on well and wash with 2.5ml EQ2 (**EB 10ml +225mg Iodoacetamate + 50ul Bromofenol Blue** (to follow proteins) 10min with shaking.

Prepare 1x TGS Buffer (Bio Rad) (500ml): 50ml 10x TGS + 450ml mqH_2O. 50ml in Falcon

33. Take off cones from the gels (be careful).
34. Put gels on a bucket (small face to the interior). Add a little quantity of 1x TGS Buffer to check. Put the bucket inside the cube.
35. Add 1x TGS to cover the gels.
36. Dry the strip (careful! They're sticky) in paper and wash in 1x TGS (falcon 50ml) three times.
37. Insert the strip inside the staking gel (gel to the interior, so put the pH name before and let to fall). + end of the strip in the red electrode.
38. Add 5ul Prot MW to the small well in the lateral – near to the pH name of the strip.
39. 2D: 120v, constant – 60min – 4ºC in cold room or Cube inside ice.
40. Remove small face with spatula. Remove staking gel and take the gel.
41. Put it on Instant Comassie (staining step) 20 min 35rpm.
42. Put gel over a white platform.

Media Recipes

MEDIA TO GROW

SD medium (1L)
40mL YNB 20X (w/o aa w $(NH_4)_2SO_4$, si no tiene añadir 20g Amonium Sulphate)
910mL H_2Od
(Autocleavage)
50mL Glc 40% (esterilizado aparte)

The reference strain BY4743 is auxotrofic for URA, LEU and HIS.Add to SD media (100mL): 1ml URA, 0.2ml HIS, 1ml LEU of the stock.
For 1L: 10ml URA, 2ml HIS, 10ml LEU.

Stock:
Uracil: 0.2g/100ml
Leu: 1g/100ml
His HCl: 1g/100ml

SD (synthetic dextrose) minimal medium:

Bacto yeast nitrogen base without aa (0.67%):	6.7g
Glucose (2%)	20g
Bacto-agar (2%)(optional)	20g
H_2Od	1L

We have YNB prepared so add 40ml to 910 H_2Od (Autocleavage) + 50ml Glc (prepared before steril by autocleavage).

BUFFERS

YEB 500mL Stock.
100mL 0.5M HEPES-KOH
9.13g KCl (1M final [ ])
1mL 0.5M EGTA (1mM)
1mL 0.5M EDTA (1mM)
H_2Omq 394.75
(Sterilized by filtration).
1.25mL 1M DTT (2.5mM) (Fisher Scientific).
1mL 0.1M PMSF (Phenylmethanesulfonyl fluoride 99% Sigma).
Bold components are prepared before and sterilazed by filtration. The other components are added before using the buffer.

HEPES-KOH 500mM (250mL) pH 7.9
29.7g HEPES
Add KOH until pH 7.9

YEB ready to use.
40ml YEB stock + 100ul DTT (Stock: 500ul mqH_2O + 77mg DTT) + 80ul PMSF (Stock 500ul EtOH 100% + 8.7mg PMSF) + 1 EDTA free-tablets.

PROTEIN SOLUTION (Lysis buffer) 50mL Stock

8M Urea	24g
50 mM DTT (Dithiothreitol)	385mg
4% CHAPS	2g
2% Carrier Amph (Bio Rad)	1mL each 4/6 and 5/7
2 EDTA free tablets	2 tablets

(Check the content of EDTA free tab)

Aliquot in eppendorf 1ml to avoid to defrost several times.

EQUILIBRATION BUFFER **(EB)** (100ml):

Urea	36g
SDS 20% (Bio Rad)	10ml
Tris-HCl 1.5M pH 8.8	3.3ml
Glicerol 50%	40ml

Up to 100ml mqH_2O

MAKING GELS

SMALL GEL (follow the order):

12% Running gel (20ml):

40% Acrylamide	6ml
1.5M Tris-HCl pH 8.8	5ml
10% SDS	0.5ml
H_2Omq	8.7ml
TEMED (In flow cabinete)	10ul
10%APS (Ammonium persulphate)	100ul (BioRad: 0.1g + 900ul mqH_2O).

4%Stacking gel (5ml):

40% Acrylamide	0.5ml
0.5M Tris-HCl pH 6.8	1.25ml
10%SDS	50ul
H_2O	3.2ml
TEMED	5ul
10%APS	25ul

BIG GEL:

12% Running gel (250ml):

40% Acrylamide	45ml
1.5M Tris-HCl pH 8.8	37.5ml
10% SDS	3.7ml
H_2Omq	63ml
TEMED	75ul
10%APS	750ul

4%Stacking gel (25ml):

40% Acrylamide	2.5ml
0.5M Tris-HCl pH 6.8	6.25ml
10%SDS	250ul
H_2O	16ml
TEMED	25ul
10%APS	125ul