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Preparation of nuclear extracts (for confluent cells in 10cm dish)

1. Wash cells with cold PBS and then harvested with cold PBS containing 1mM EDTA.
2. Centrifuge (5 minutes at 6000 rpm) and then resuspend cells in 500 μl of buffer A (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1 mM EGTA, 0.5 mM PMSF, 1mM DTT and 10 μg/ml of each of the protease inhibitors leupeptine, aprotinine and pepstatine).
3. Allow cells to swell on ice for 15 minutes before addition of 50 μl of NP-40, followed by vigorous vortexing.
4. After centrifugation, the supernatant (cytosolic fraction) is set aside and the nuclear pellet is resuspended in 100 μl of buffer B (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.4 M NaCl, 5% glycerol, 0,5 mM PMSF, 1mM DTT, and 10 μg/ml of each of the protease inhibitors) and shaken vigorously at 4°C for 30 minutes.
5. Centrifuged the extract (max speed for 15 minutes) and the protein concentration (nuclear fraction) of the supernatant is estimated by Bradford assay.

Western blots

A. <u>Gel</u> :

Acrylamide 29:1.
Deionized with AG501-X8 (Can be conserved for 6 months at 4 °C protected from light)

Resolving buffer 4X (lower):
Tris 1.5M pH8.8
SDS 0.4%

Stacking buffer 2X (upper):
Tris 0.25M pH6.8
SDS 0.2%

Ammonium persulfate (APS) 10% (keep at 4 °C for a week)

TEMED

n-butanol saturated with H_2O

SDS 10%

10X Tris-glycine buffer
250 mM Tris
2.5 M Glycine
1L stock solution (keep at 4 °C): 30g of Tris base + 143.75g of glycine

Tris-glycine electrophoresis buffer
25 mM Tris
250 mM glycine
0.1% SDS
Make 1L: 100mL of Tris-glycine 10X + 10 mL SDS 10%

LAEMLLI 2X buffer
100 mM Tris.Cl pH 6.8
4% SDS
20% glycerol
0.2% bromophenol blue
Conserve at room temperature. BEFORE use, add 1 part DTT 1M to 4 parts of LAEMLLI buffer (final concentration of DTT= 200mM)

B. Transfer :

Transfer buffer

25 mM Tris
250 mM glycine
20% methanol
Make 1L: 100 mL of Tris-glycine 10X + 200 mL of methanol 100%

C. Western and detection by chemiluminescence :

TBS 10X

Tris.Cl 100mM pH 7.5
9% NaCl

TTBS

1X TBS + 0.1% Tween-20 (polyoxyethylenesorbitan monolaurate)

ECL+ chemiluminescence detection kit (Amersham, RPN 2133)

A. Gel protocol

1. Assemble Mini-Protean gel (Bio-Rad) with 1.5 mm spacers (for samples up to 50uL). Make sure the glasses are clean and use EtOH so gel won't stick.

2. Prepare resolving gel (in mL, total 10mL)

	7.5%	10%	12%
Resolving buffer 4X	2.5	2.5	2.5
Acrylamide 29:1	2.5	3.3	4
APS 10%	0.1	0.1	0.1
TEMED	0.01	0.01	0.01
ddH2O	5	4.1	3.4

The concentration of acrylamide depends on the range of separation you are looking for:

Acrylamide concentration (%)	Linear range of separation (kD)
5	57 to 212
7.5	36 to 94
10	20 to 80
12	12 to 60
15	10 to 43

3. Pour resolving gel allowing +/- 2cm at the top for the stacking gel and the comb. Immediately cover it with saturated n-butanol (it's the upper phase!) so the upper part of the gel polymerizes in a straight line. Polymerization takes 15-20 minutes.

4. Once the gel is polymerized, wash away the n-butanol by rinsing with ddH_2O

5. Pour stacking gel and place comb immediately as polymerization is RAPID.

	Stacking gel (in mL, 5 mL total, 4%)
Stacking buffer 2X	2.5
Acrylamide 29:1	0.66
APS 10%	0.1
TEMED	0.01
ddH2O	1.8

6. Prepare the molecular markers. Typically, take 10 uL of marker and add 10 uL of LAEMLLI 2X (containing DTT) - DO NOT BOIL!!!!!

7. Prepare the samples. Add 1 volume of LAEMLLI 2X (containing DTT) to 1 volume of sample for a max of 50 uL.

8. Boil immediately for 5 minutes and then keep on ice

9. Load samples and run gel at 190V, 45-60 minutes or until the bromophenol blue runs out of the gel…

B. Transfer protocol

1. While gel is running, prepare the transfer buffer. Keep approximately 800 mL at -20°C so it cools before the actual transfer.

2. Cut 2 pieces (a little bigger than the actual gel) of 3M Whatman paper and the PVDF membrane (Millipore Immobilon-P transfer membrane, pore size 0.45 um).

3. Soak PVDF membrane in 100% methanol for 2 min, then in water for an additional 2 min and finally in transfer buffer until the assembly of the "transfer sandwich".

4. After running the gel, cut the stacking gel and the excess acrylamide. Wash the gel twice at room temperaure for 15 minutes in 75 mL of transfer buffer with agitation (this gets rid of the SDS in the gel, otherwise it will precipitate at 4°C and interfere with the detection).

5. Assemble the "transfer sandwich":

    -
    humidified sponge
    humidified Whatman paper
    gel
    PVDF membrane
    humidified Whatman paper
    humidified sponge
    +

6. Place in transfer apparatus, making sure the POLARITY is correct (the PVDF membrane on the + side!). Add the cooled transfer buffer and a stir bar.

7. Tranfer at 4°C with magnetic agitation for 1 hour at ~100V. Temperature can get high so check frequently.

8. After transfer, place the membrane in TTBS/5% skimmed milk for 30 minutes at RT or overnight at 4°C.

C. Detection protocol

1. Incubate with primary antibody diluted in TTBS/5% skimmed milk for 2 hours at room temperature or overnight at 4°C. The dilution is determined empirically (I typically start with 1:1000 dilution).

2. Wash 3 x 10 minutes with TTBS

3. Incubate with appropriate secondary antibody COUPLED with HRP (horseradish peroxidase) to allow direct detection without amplification step. Incubate for 1 hour at room temperature. Dilute the antibody in TTBS/5% skimmed milk (reduces background). Dilution is determined empirically (typically 1:20000 for anti-mouse-HRP, 1:50000 for anti-rabbit or chicken-HRP)

4. Wash 3 x 10 minutes with TTBS

5. Detection with a chemiluminesence kit (such as the ECL+ kit from Amersham) following the manufacturer specifications.