WESTERN BLOT

Reagents:

Lysis Buffer (per mL)

917 uL TPER Reagent (Pierce # 78510)

60 µL 5 M NaCl (300 mM final concentration)

10 µL of 100 mM orthovanadate (1 mM final conc.) To obtain a 100 mM solution of ortovanadate add 0.01839 g of ortovanadate in 1.0 ml dH_2O. Then, boil the solution for 10 minutes.

10 µL 200 mM PEFABLOC (AEBSF) (Roche # 1 585 916)

1 µL 5 mg / mL Aprotinin (5 µg / mL final concentration) (Sigma cat. # A1153)

1 µL 5 mg / mL Pepstatin A (5 µg / mL final concentration) (Sigma cat. # P5318)

1 µL 5 mg / mL Leupeptin (5 µg / mL final concentration) (Sigma cat. # L9783)

Note: the final concentration of NaCl is 450 mM as the TPER contains 150mM of NaCl.

Procedures:

Reconstitution of protease inhibitors

A. Aprotonin: resuspend at a concentration of 5.0 mg/mL in dH_2O. Aprotonin is stable for 6 months at -20°C.

B. Pepstatin A: resuspend at a concentration of 5.0 mg/mL in 10% acetic acid in methanol. Pepstatin A is stable for 6 months at -20°C.

C. Leupeptin: resuspend at a concentration of 5.0 mg/mL in dH_2O. Leupeptin is stable for 6 months at -20°C.

D. PEFABLOC (AEBSF): resuspend at a concentration of 200 mM in dH_2O. PEFABLOC is stable for 2 months at -20°C.

239.5 mg / mL = 1.0M

47.9 mg / mL = 200Mm

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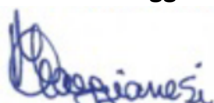


Cell Lysis and Protein Quantification:

1) Store the pellets in ice, centrifuge for 2 minutes at 1500 rpm in a previously refrigerated centrifuge (4°C) and remove the supernatant.

2) Resuspend the pellet in an appropriate volume of lysis buffer, vortex for 15 seconds and incubate in ice for 30 min; after 15 minutes repeat the vortex operation. Volume of the lysis buffer: 1×10^6 cells/100 µl of lysis buffer.

3) Centrifuge for 10-15 minutes at 13000 rpm and transfer the supernatant into a new tube.

4) Quantify the proteins obtained using the Bradford method (1 microliter of lysate in a ml of bradford solution) and read to the spectrophotometer at a wavelength of 595 nm.

5) Dilute the sample with SAMPLE BUFFER SDS 2X + 5% β-mercaptoethanol. For RPPA resuspend 25 migrograms of sample in 2X SDS sample buffers in a final volume of 50 microliters.

6) Boil the samples at 4°C for 4 minutes. Proceed with loading or store the sample at -80°C.

Protein extraction from frozen tissue

1) Prepare the "UltraTurrax" workstation, mount the previously autoclaved tip and prepare three containers containing %SDS, mM NaOH and dH_2O.

2) Transfer the piece into a cytofluorimetry tube, prepare the lysis buffer as previously described and provide homogenization of the sample by ultraturrax. After each sample, carry out washings in NaOH, SDS, distilled H_2O and dH_2O milliQ.

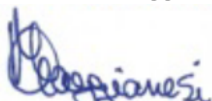
3) Proceed with the extraction protocol previously described.

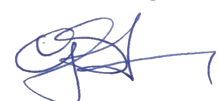
Preparation and loading of polyacrylamide gel

1) Prepare the polyacrylamide gel according to the following table:

RESOLVING (Lower part)		
Solution components	**10mL**	**20mL**
8%		
H20	4,6	9,3
30% Acrylamide mix	2,7	5,3
1,5 M Tris (pH 8.8)	2,5	5
10% SDS	0,1	0,2
10% APS (0,1g in 1mL H20)	0,1	0,2
TEMED	0,01	0,02
10%		
H20	4	7,9
30% Acrylamide mix	3,3	6,7
1,5 M Tris (pH 8.8)	2,5	5
10% SDS	0,1	0,2
10% APS (0,1g in 1mL H20)	0,1	0,2
TEMED	0,01	0,02
15%		
H20	2,3	4,6
30% Acrylamide mix	5	10
1,5 M Tris (pH 8.8)	2,5	5
10% SDS	0,1	0,2
10% APS (0,1g in 1mL H20)	0,1	0,2
TEMED	0,01	0,02
STACKING (Upper part)		
Solution components	**3 mL**	**6 mL**
H20	2,1	4,1
30% Acrylamide mix	0,5	1
1 M Tris (pH 6.8)	0,38	0,75
10% SDS	0,03	0,06
10% APS (0,1g in 1mL H20)	0,03	0,06
TEMED	0,006	0,012

2) Mount the gels in the appropriate travel chamber, taking care to place the smallest glass always inside and fill with running buffer. After boiling the samples, load the samples using the appropriate tips. Run the gel up to a maximum of 100V until the samples enter the "*separating*" and subsequently increase up to a maximum of 140V.





Running buffer recipe:

RUNNING BUFFER 5X	
SOLUTION COMPONENTS	**Quantità**
Trizma base	15,1 g
Glicina	94 g
SDS 20%	25 mL
dH2O	Portare a volume di 1L

Polyacrylamide gel transfer

1) Before the running of the gel finishes, prepare everything necessary for nitrocellulose membrane transfer.

N.B. For proteins up to 110 kDa a semi-dry transfer can be used, whereas for high molecular weight proteins it is necessary to use immersion transfer.

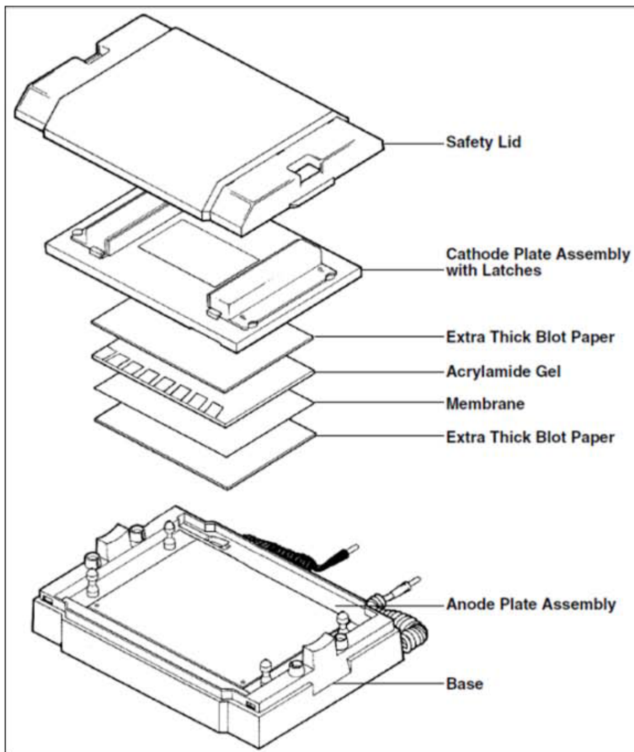
- Cut the sponges and the membrane (9 cm x 6 cm), activate the latter for 30 seconds in dH_2O and equilibrate it for 10 minutes in transfer buffer.

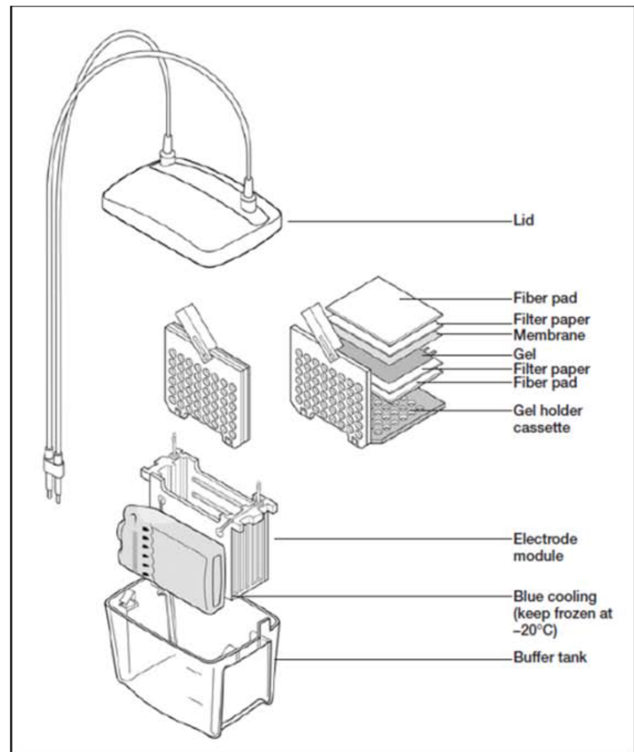
Transfer buffer recipe:

TRANSFER BUFFER	
SOLUTION COMPONENTS	**Quantità**
Trizma base	5,8 g
Glicina	2,9 g
SDS 20%	1,85 mL
Metanolo	200 mL
dH2O	Portare a volume di 1L

2) At the end of the run, equilibrate the gel for about 10 minutes in the transfer buffer and proceed with the preparation of the transfer "*sandwiches*".

Semi-dry transfer **Immersion transfer**





3) Transfer to 15V for 45 minutes and then to 20V for 5 minutes for the semi-dry transfer, whereas transfer to 30V O.N. in ice for the immersion transfer.

4) At the end of the transfer check the efficiency by staining the membrane with red ponceau: immerse the membrane for a few seconds and rinse with plenty of distilled H_2O and TBS-T buffer.

TBS-T buffer recipe:

TBS BUFFER 5X	
SOLUTION COMPONENTS	**Quantità**
Trizma base	12,1 g
NaCl	40 g
dH2O	Portare a volume di 1L
pH 7,4	

N.B. Add 1mL of Tween 20 to the 1X solution (TBS-T).

5) Proceed to membrane blocking using a 5% non-fat dry milk solution in TBS-T and shake gently the membrane for approximately 45 minutes. Then, rinse with TBS-T and proceed with the primary antibody incubation.

Note: Titration experiments must be performed to determine the exact concentration to be used for the antibody and the amount of protein to be loaded.

Incubation of primary antibody and development

1) After carrying out the Blocking of the membrane, incubate the same O.N. with the primary antibody to the chosen concentration.

2) The next day incubate the membrane at room temperature for about 30 minutes, recover the primary antibody (add sodium azide and store the antibody at the storage temperature of the *mother* antibody) and perform three washings with TBS-T.

3) Incubate the membrane with a 5% milk solution in TBS-T containing the specific secondary antibody (mouse, rabbit, goat, etc) for 45 minutes.

4) Perform three washes with TBS-T.

5) Prepare the development solution (SuperSignal™ West Dura Extended Duration Substrate) and develop the membrane using CHEMIDOC GE HEALTCARE or LYCOR SYSTEM.

6) If a membrane has to be incubated with a different primary antibody, incubate the membrane at 37°C with stripping solution (Thermo scientific); perform three washings with TBS-T, the membrane blocking using a solution of non-fat dry milk at 5% in TBS-T, and proceed with the incubation procedure previously described.