PROTOCOLS FOR GEL ELECTROPHORESIS AND WESTERN BLOTTING

These protocols are used for current practice in many laboratories. The standard protocol is followed by minor modifications used in other laboratories. All of them have been tested with good results, and preferences are largely dependent on personal feelings.

SAMPLE HOMOGENIZATION

Approx. 0.1 grs of fresh tissue in 4 volumes of buffer:

* Lysis buffer: 20 mM HEPES-KOH pH = 7.5

  250 mM sucrose

  10 mM KCl

  1.5 mM MgCl2

  1 mM EDTA

  1 mM EGTA

  1mM DTT

  0.1 mM PMSF

  a table of proteases inhibitors (Roche) in 10 mL of lysis buffer

* Centrifuge 15,000xg 5 min.

* Remove the supernatant carefully

PROTEIN QUANTIFICATION: BRADFORD (1l)

0.1 grs Comassie Brilliant

12.5 ml ethanol 95º

100 ml phosphoric acid at 85%

850 ml H2O miliQ

Once the protein is quantified from the supernatant, dilute in sample buffer at a ratio of 1:1

BrainNet Europe II

Project Co-ordinator:

Prof. Dr. Hans Kretzschmar

Contact:

www.brainnet-europe.org

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Content

SAMPLE BUFFER (2X)

1.514 grs Tris Base in 25 ml miliQ H2O (125mM), adjusted pH=6.8 with HCl

40 ml SDS 10%

20 ml glycerol

0.002 grs Bromophenol Blue

Add miliQ H2O until 100 ml

β-mercaptoethanol at 10% is added to the samples just before used.

Heat the sample at 95% for 5 min. Keep at -20ºC until use.

ELECTROPHORESIS

RESOLVING SOLUTION

	8%	10%	12%	15%
Acrylamide	2.66ml	3.33ml	4ml	5ml
Buffer R	2.5ml	2.5ml	2.5ml	2.5ml
H_2O	4.69ml	4.02ml	3.35ml	2.35ml
Temed	10µl	10µl	10µl	10µl
AP	100µl	100µl	100µl	100µl

(Acrylamide: Bio-rad, 30%, 29:1)

STACKING SOLUTION

	4%
Acrilamide	1.3ml
Buffer S	2.5ml
H_2O	6.1ml
Temed	10µl
AP	100µl

Buffer R: 1.5M Tris HCl PH= 8.8

0.1% SDS

Buffer S: 0.5M TrisHCl PH= 6.8

AP: Ammonium Persulfate: 0.1 mgrs/ml H2O miliQ

RUNNING BUFFER (10x)

30.28 grs/l Tris (0.25M)

14.13 grs/l glycine (1.92M)

10 grs/l SDS (0.1%)

Conditions: constant current, 0.02A per gel

SEMI-DRY TRANSFER

4 whatman papers + nitrocellulose membrane + gel + 4 whatman papers

(all embedded in semi-dry buffer)

Conditions: constant current, 0.04A per gel, 45 minutes.

SEMI-DRY BUFFER (500ml)

2.9 grs Tris

1.45 grs glycine

100 ml methanol

0.19 grs SDS

Alternatively (depending on the molecular weight of the protein):

SANDWICH TRANSFER

Sandwich buffer

250 mM Tris base

92 mM glycine

in 1 l of distilled water.

Add 10% of methanol (if proteins <30 kDa) or 20 % (if proteins >30 kDa) just before use.

(This protocol has been prepared for using the Mini-Trans blot Electrophoretic Transfer

cell (Bio-Rad))

- Fill the Bio-Ice cooling unit with water and store it in your laboratory freezer at -20 °C until ready to use. After use, return the cooling unit to the freezer for storage.
- All electrophoresis gels should be pre-equilibrated in transfer buffer prior to electrophoretic transfer. Pre-equilibration will facilitate the removal of contaminating electrophoresis buffer salts and neutralization salts (salts resulting from the denaturation of nucleic acids prior to transfer). If the salts are not removed, they will increase both the conductivity of the transfer buffer and the amount of heat generated during the transfer.

Protocol:

1. Prepare the transfer buffer. (Using buffer chilled to 4 °C will improve heat

dissipation.)

2. Cut the membrane (a) and the filter paper to the dimensions of the gel. Always wear gloves when handling membranes to prevent contamination. Equilibrate the gel and soak the membrane, filter paper, and fibre pads in transfer buffer (15 min–1 hour, depending on gel thickness).

3. Prepare the gel sandwich. Place the cassette, with the grey side down, on a clean surface.

Place one pre-moistened fibre pad on the grey side of the cassette.

Place a sheet of filter paper on the fibre pad.

Place the equilibrated gel on the filter paper.*

Place the pre-moistened membrane on the gel.*

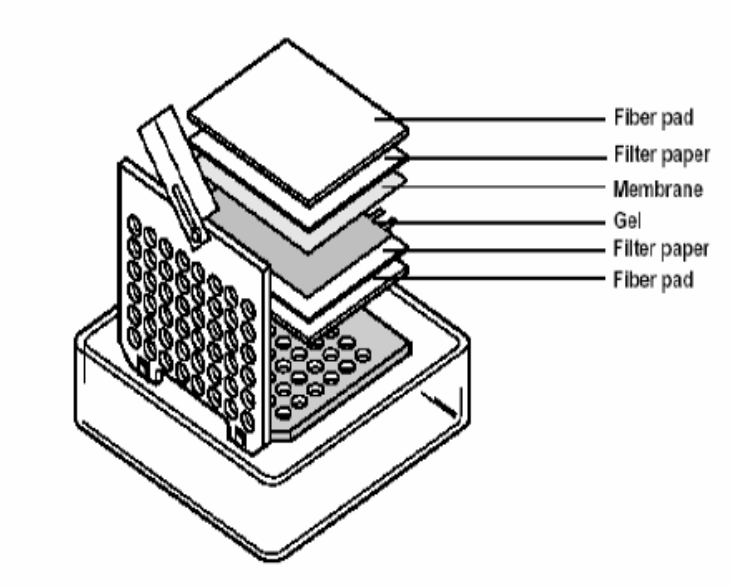
Complete the sandwich by placing a piece of filter paper on the membrane.*

Add the final fibre pad.

* Removing any air bubbles which may have formed is critical for good results. Use a

glass tube to gently roll air bubbles out.

(a) Nitrocellulose or PVDF membranes can be used. PVDF (Polyvinylidene difluoride) membranes must first be moistened in 100% MetOH.



4. Close the cassette firmly, being careful not to move the gel and filter paper sandwich.

Lock the cassette with the white latch.

5. Place the cassette in the module. Repeat for the other cassette.

6. Add the frozen Bio-Ice cooling unit. Place in tank and completely fill the tank with buffer.

7. Add a standard stir bar to help maintain even buffer temperature and ion distribution in the tank. Set the speed as fast as possible to keep ion distribution even.

8. Put on the lid, plug the cables into the power supply, and run the blot. Transfer conditions: 200 mA per gel for 90 min. Voltage must not exceed 120V.

9. Upon completion of the run, disassemble the blotting sandwich and remove the membrane for development. Clean the cell, fibre pads, and cassettes with laboratory detergent and rinse well with de-ionized water.

*When the membrane is transferred, check the transference efficiency with Ponceau staining. Mark the molecular weight standards and rinse the membrane with distilled water to take out the dyer.

IMMUNOBLOTTING

Reagents

1. TBST 10X (Tris-Buffered Saline) buffer

Trizma base 12.11 g

NaCl 81.81g

dH2O 1000 mL

Adjust pH to 7.4 with HCl

Add 1% Tween-20.

2. Blocking solution

5% skimmed milk in TBST

3. Primary antibody

Dilute the primary antibody with TBST-BSA3%

4. Secondary antibody (prepare before use)

Dilute the secondary antibody 1:1000 with blocking solution

Protocol

1. Block the membrane for 1h with blocking solution at room temperature.
2. Wash the membrane with TBST 3 times, 5 minutes each.
3. Incubate the membrane with the primary antibody at the appropriate dilution overnight at 4ºC.
4. Repeat step 2.
5. Incubate the membrane with the secondary antibody for 45 minutes at room temperature.
6. Wash the membrane with TBST for 30 minutes as a minimum, changing the buffer each 5 or 10 minutes. Longer washing times will increase the efficiency of the detection.

7. Different detection methods can be used, depending on the sensitivity and the specificity of the antibodies used. In general a chemiluminescent method is used to detect the reactive bands following the instructions given by the manufacturer.

MEMBRANE STRIPPING

To re-utilize the membranes with another primary antibody

Stripping buffer

3.78 grs of Tris in 500 ml distilled H2O. Adjust pH=6.8 with HCl

10 grs SDS (2%)

3.48 ml β-mercaptoethanol

Wash membranes with TBST

Incubate the membranes twice for 20 min. each time with stripping buffer at 66ºC, approx.

3 washes for 5 min. each of TBST

Repeat the immunoblot from blocking of the non-specific unions.

MODIFICATIONS TO THE STANDARD PROTOCOL

PROTOCOL EDINBURGH

Protein Extraction and Quantification

- Approx. 0.1g frozen brain tissue was placed into sterile eppendorfs containing 1mL lysis buffer:
- Lysis Buffer:
  - o 20 mM HEPES-KOH, pH 7.5
  - o 250 mM sucrose
  - o 10 mM KCl
  - o 1.5 mM MgCl2
  - o 1 mM EDTA
  - o 1 mM EGTA
  - o 1 mM DTT

o 0.1 mM PMSF

- Sigma Protease Inhibitor Cocktail (10 l) was added to each sample.
- Samples were lysed at 4oC for 1 hr and were vortexed intermittently.
- Centrifuge at 13,000 rpm for 20 min and remove supernatant to a new sterile eppendorf.
- Samples were diluted 1:1 in sample buffer and heated at 95oC for 5 min prior to use.
- Sample Buffer:

  o 1.25 ml 0.5M Tris-HCl, pH6.8

  o 2.5 ml glycerol

  o 2 ml 10% SDS

  o 0.2 ml 0.5% Bromophenol Blue

  o 3.55 ml dH2O

  Add 50 µL mercapto to 950 µL buffer prior to use.

Electrophoresis

- 10% resolving gel and 4% stacking gel on Mini Protean 3 system from Biorad. However, was run at 100V, constant current
- High Molecular Weight Rainbow Markers (Amersham)
- Approx. 50 g protein per well.

Sandwich Transfer

- Was run using Mini TransBlot Electrophoretic Transfer Cell (Biorad)
- 2 filter pads + gel + PVDF (Immobilon) + 2 filter pads
- Sandwich buffer with 20% methanol
- Transferred at 100V for 1 hr

Immunostaining

- Membrane was equilibrated in TBST for 15 min then blocked in TBST-4% BSA for 1 hr on a rocker, RT
- Primary antibody (b-actin 1:5000; phosphor-AKT 1:1000) was added and incubated at 4oC overnight on a rocker.
- 3 x 10 min washes in TBST-4% BSA
- Biotinylated secondary antibodies were added at 1:2000 (b-actin – rabbit anti mouse, DAKO; phosphor-AKT- swine anti-rabbit, DAKO) and samples incubated at RT for 1 hr.
- 1 x 10 min wash in TBST-4% BSA, 2 x 10 min washes in TBST
- Streptavidin-ABC (DAKO) diluted 1:50 was added and samples incubated for 30 min at RT
- 3 x 10 min washes in TBST
- Develop with Vector VIP peroxidase substrate kit for 15 min. Wash in dH2O for 5 min. Dry membranes.

WESTERN BLOT: PROTOCOL MILANO

Lysis buffer normally used:

NaCl 100mM

EDTA 10mM

NP40 0.5%

NaDeoxic 0.5%

Tris pH7.4 10mM

PEFABLOC 1mg/ml

EDTA 0.5mg/ml

LEUPEPTINE 10µg/ml

PEPSTATINE 10µg/ml

APROTININE 1 µg/ml

Centrifugation 15,000xg 5 min.

Remove the supernatant carefully

PROTEIN QUANTIFICATION: BCA PROTEIN ASSAY (PIERCE)

0.1 grs Comassie Brilliant

12.5 ml ethanol 95º

100 ml phosphoric acid at 85%

850 ml H2O miliQ

Once the protein is quantified, dilute in sample buffer at a ratio of 1:1

SAMPLE BUFFER (2X)

SDS 4% → 40ml

TRIS pH6.8 120mM → 1.454g

BB 0.002% → 0.002g

GLYCEROL 20% → 20ml

DW until 100ml

DTT 100mM

Heat the sample at 95% for 10 min. Keep at -20ºC until use.

ELECTROPHORESIS

RESOLVING

	8%	10%	12%	15%	17%	15%	12.5%	10%	7.5%
Acrylamide	2.66ml	3.33ml	4ml	5ml	25.5ml	22.5	18.75	15	11.25
Buffer R	2.5ml	2.5ml	2.5ml	2.5ml	Tris SDS	pH8.8 20%	1M	16,8ml 225µl	
H_2O	4.69ml	4.02ml	3.35ml	2.35ml	2.85ml	5.55	9.3	13	16.8
Temed	10µl	10µl	10µl	10µl	30 µl				
AP	100µl	100µl	100µl	100µl	150 µl				

(Acrylamide: Bio-rad, 30%, 29:1)

STACKING

	4%	5% (for 4gels)
Acrilamide	1.3ml	5ml
Buffer S	2.5ml	Tris pH6.8 1M 3.8ml SDS 20% 150 µl
H_2O	6.1ml	21ml
Temed	10µl	15µl
AP	100µl	150µl

Buffer R: 1.5M Tris HCl PH= 8.8

0.1% SDS

Buffer S: 0.5M TrisHCl PH= 6.8

Ammonium Persulfate: 0.1 mgrs/ml H2O miliQ

RUNNING BUFFER (10x)

30.28 grs/l Tris (0.25M)

14.13 grs/l glycine (1.92M)

10 grs/l SDS 1%

Conditions: constant voltage

SEMI-DRY TRANSFER

4 whatman papers + PVDF membrane + gel + 4 whatman papers

(all embedded in semi-dry buffer)

conditions: constant current

SEMI-DRY BUFFER (500ml)

2.9 grs Tris

1.45 grs glycine

100 ml methanol

0.19 grs SDS

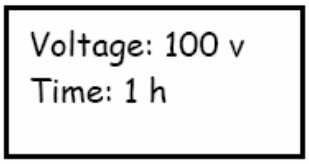
Alternatively (depending on the molecular weight of the protein):

SANDWICH TRANSFER

2 whatman papers + gel + nitrocellulose membrane + 2 whatman papers

(all embedded in sandwich buffer)

Transfer conditions



TBST BUFFER (10x)

12.11 grs (100 mM) Tris

81.81 grs (1.4 M) NaCl

Adjust pH= 7.4 with HCl and then add 1% Tween-20

Add miliQ H2O to 1 l

Blocking of non-specific unions: 1 h with TBST+ 5% skimmed milk

Primary antibody inTBST-milk. Overnight at 4ºC

3 washes for 10 min in TBST

Secondary antibody diluted in TBST-milk at 1:3000 (DAKO) for 60 min

3 washes for 10 min in TBST

3 washes for 5 min. each in TBST

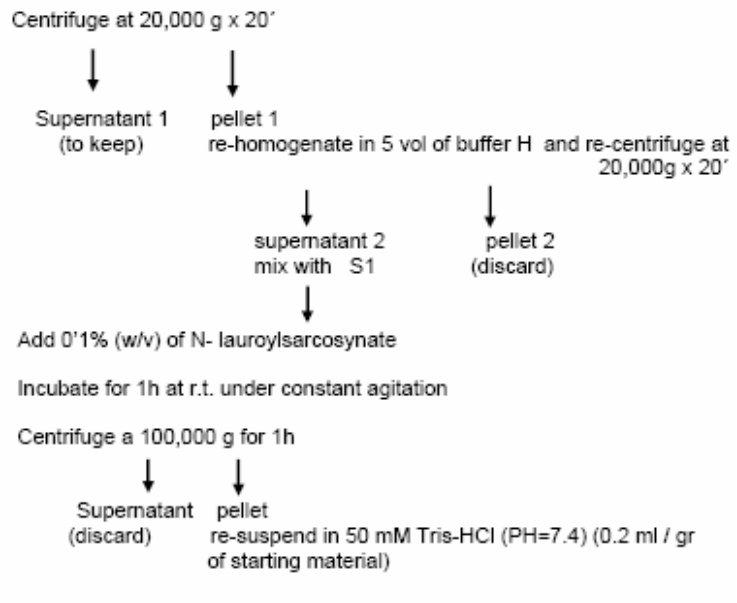
Develop with ECL (Amersham)

PHF PURIFICATION PROTOCOL

Protocol used to obtain cellular fractions enriched in paired helical filaments and related filaments in Alzheimer's disease and tauopathies

Tissue homogenisation (approx 2-3 grs of tissue) 1/10 (w/v)

Buffer H: 10mM Tris-HCl (PH= 7.4)
0.8M NaCl
1mM EGTA
10% sucrose
1mM PMSF
protease inhibitor cocktail



Determination of the protein concentration with BCA method (Pierce)

Electrophoresis (load approx.100 µgrs of protein) and Western blot.

TWO-DIMENSIONAL GEL ELECTROPHORESIS

Brain tissue (0.1g) suspended in 1 mL of extraction buffer consisting of 40 mM Tris-HCl, 7M urea, 2M thiourea, 4% CHAPS and a mixture of protease inhibitors (Sigma). The suspension is sonicated for approximately 30 sec (except if the sample is prepared for oxydized proteins) and centrifuged at 12,000 rpm for 15 min. Sonication can produce a kind of oxidative stress, exaggerating the final results. The protein content in the supernatant is determined with the Bradford method. 300 µg of protein sample are diluted in extraction buffer plus 2 mM TBP (Tributylphosphine) and 0.2% carrier ampholytes pH 3-10 applied (300 µL) on immobilized pH 3-10 linear gradient strips (17 cM). The re-hydration step is done overnight passively. The proteins are focused at 300 V for 1h, after which the voltage is gradually increased to 3,500 V within 6 h. Focusing is continued at 3,500 V for 12 h and at 5,000 V for 24 h. Before the second dimension starts, the strips must be equilibrated first in equilibration buffer plus 2% DTT for 20 minutes, and afterwards in the same buffer but now containing 2.5% iodoacetamide, for 20 min. Finally, the strip is soaked in SDS-PAGE running buffer for 30 sec and then placed onto the gel. The second-dimensional separation is usually performed on 10% polyacrylamide gels at 200 V for 4-5 h. After protein fixation with 40% methanol containing 10% acetic acid in de-ionized water for 1h as a minimum, the gels are stained with the Silver Staining kit (Amersham-Pharmacia), following the protocol supplied by the manufacturer.

DETAILED 2D PROTOCOL

1. PROTEIN PRECIPITATION

Buffer interference with the first dimension must be avoided. A way to achieve this is by protein precipitation followed by solution in 2D-sample buffer.

Precipitation buffer (PB)

Tris-Bufferd Saline (TBS) buffer 10mM pH 7.4.

Ethylenediaminetetraacetic (EDTA) 1mM

Sodium Pyrophosphate 5mM

β-glycerolphosphate 30 mM

Sodium Fluoride 30 mM

(May be prepared and kept at –20ºC)

(The starting amount is not important. What is important is the ratio of sample to PB).

1. Homogenize 0.1g of brain sample in 4-5 volumes of PB.

The amount of protein is quantified by the BCA method using BSA as a standard. The same amount of protein from each sample is used to precipitate.

2. Four volumes of HCl 2N are added to each sample and incubated for 20 minutes at room temperature.

3. Tri-chloroacetic acid (TCA) at 15% final volume is added to each sample and incubated on ice for 10 minutes.

4. Vortex the samples to mix well.

5. Centrifuge 15,800 g, 2 minutes. Supernatants are to be discarded.

6. Wash the pellets 3 times in ethanol-ethylacetate (1:1). These final pellets are the precipitated proteins which are resuspended in 300µL of sample buffer.

2. 2D-ELECTOPHORESIS

Material

Protean IEF system

Cup loading tray set

Protean IEF cell

Protean II XI basic unit, casting stand

Protean II XI cell IPG conv. kit

IPG ready strip pH 3-10 7 cm

IPG ready strip pH 3-10 17 cm

100x Bio-Lyte 3-10 Ampholyte, 1 mL

Sample buffer (sb)

Tris-HCl 40mM, pH 7.4

Urea 8M

Thiourea 7M

Tributhylphosphine (TBP) 2mM

CHAPS 4%

Ampholytes 0.2%

Bromophenol blue 0.0002%

Sample preparation

Samples must always be dissolved before running in 2D-electroforesis, independently of the previous treatment. Cationic detergents must be avoided and low-salt buffers must be used to interfere as little as possible with the focusing of the sample in the first dimension.

Starting material:

a. Precipitated proteins: dilute in 300µL of SB*

b. Brain sample:

b.1. Sample homogenization in SB without bromophenol blue and without ampholytes. The ratio sample: SB described for the precipitation protocol is mantained.

b.2. Centrifuge 12,000 rpm for 15 min at 4ºC. (Some proteins may be lost during this step. The speed of the centrifuge must be adapted depending on the localization (nuclear or cytosolic) of the studied proteins).

b.3. Discard the pellet.

b.4. The amount of protein from the supernatant is quantified with the Bradford method, using BSA as a standard. * (The amount of protein to be loaded in each strip depends on the length of the strip, with 300 µg of protein as a minimum, in 300µL of final volume onto 17cm IPG strips).

First measurement

To avoid contamination during the final detection steps, all the material must be kept clean with dd H2O, and all the steps must be performed wearing gloves. After dissolving the sample the first measurement must be performed. Acrylamide strips with distributed ampholytes (IPG strips) are used depending on the pI of the proteins to be separated. After distributing the protein sample all along the IPG strip and following incubation at room temperature for 1 hour (passive hydration), the focus is programmed in the IEF cell as follows: active hydration at 50V for 16 hours; 300V for 2h; 500V for 2h; 1000V for 2h; 8000V for 8h; and, finally, 8000V for 10h. Running conditions will be adapted

depending on the proteins required for study. IPG strips can be kept at –20ºC . The running of the second measurement does not need to be carried out immediately.

Second measurement

IPG Strips equilibration

Equilibration buffer (eb).

(Can be prepared and kept at –20ºC)

Urea 6M

SDS 20%

Tris/HCl 0.05M, pH 8.8

Glycerol 20%

dd water

IPG strips must be equilibrated before starting the second measurement.

The equilibration is performed by a 2-step incubation protocol.

1. 10 minutes incubation in EB plus dithiothreitol (DTT) 2% added just before being used.

2. 10 minutes incubation in EB plus iodoacetamide 2.5% instead of DTT.

After this the IPG strips are soaked in running buffer and loaded onto SDS-PAGE electrophoresis gel, the acrylamide percentage of which will depend on the molecular weight of the proteins to be detected. The best approach is to perform a gradient gel (8-15% acrylamide) to cover as many proteins as possible for a first screening, and then, depending on the results, the gradient and/or the acrylamide percentage can be adjusted depending on the proteins required for study.

DETECTION METHODS AFTER SECOND-DIMENSION ELECTROPHORESIS

After second dimension electrophoresis, acrylamide gels are treated differently depending on the method of protein detection.

- For oxidized protein detection: Western blot against anti-DNP antibody.
- For a general screening of the electrophoresis: Comassie blue staining.
- For a more accurate analysis of the samples to detect small differences between control and pathological samples, and for sequencing protein spots: Silver Staining or Cypro Ruby fluorostaining.

MASS SPECTROMETRY COMPATIBLE SILVER STAIN PROTOCOL

- Described amounts are for a 17 cm gel.
- All reagents need to be prepared in dd H2O and the protocol must be followed using glass containers.
- Procedure described following the instructions of the manufacturer: Silver Staining Kit Protein (Amersham Biosciences)

Fixing: 15 min x 2

- 40% MetOH: 100 mL
- 10% Acetic acid: 25 mL
- dd H2O: make up to 125 mL

This step can be performed O/N

Sensitizing: 30 min.

- 30% MetOH: 37.5 mL
- 5mL Sodium Thiosulphate (5% w/v)
- 8.5g Sodium Acetate
- dd H2O: make up to 125 mL

5 X 5 min washes with dd H2O (this step prevents background precipitation while silver reaction is added)

Protein Preservation

Silver reaction: 20 min

- 12.5 mL silver solution (2.5% w/v)
- dd H2O: make up to 125 mL

2 X 1 min washes in dd H2O

Developing: incubation time depends on the amount of gel.

- 3.12 g sodium carbonate
- 50 µL formaldehyde (37% w/v)
- dd H2O: make up to 125 mL

Stopping: 10 min

- EDTA-Na*2H2O (3.65g)
- dd H2O: make up to 125 mL

3 X 5 min washes in dd H2O

Author

Prof. Dr. Isidro Ferrer

Hospital de Bellvitge/
Universitat de Barcelona

Phone: +34 (93) 403 - 5808

Mail: 8082ifa@comb.es

BrainNet Europe II

Project Co-ordinator:

Prof. Dr. Hans Kretzschmar

Contact:

www.brainnet-europe.org

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Filename Protein Preservation