Western Immunoblotting—Preparation of Samples:

Total Protein Extraction from Culture Cells:

- Take off the medium
- Wash culture with 1 x PBS
- 1 ml hot Cell-lysis Solution into T75 flask
- Scrap out the cells
- Transfer lysed cells into 1.5 ml microcentrifuging tube
- Boil 10 min
- Take 100 µl out to the a new tube for protein assay
- Add 2 µl bromophenol blue into sample tube
- Store the samples in -20
  - Save lysis buffer for blank control of protein assay

Cell-lyses Solution 1:

- 1 x SDS solution + 50 mM DTT
  - add DTT freshly

10 ml of Cell-lyses Solution:
  - add 1 ml of 10 x SDS solution
  - plus 0.077 g of Dithiothreitol (DTT)
Western Immunoblotting—Preparation of Samples:

Total Protein Extraction from Tissues:

- Grind tissue in liquid nitrogen
- Label 12 x 75 cm plastic tubes and set them on ice
- Weigh tissue between 0.1 g – 0.2 g
- Add 1 ml lysis buffer with protease and phosphatase inhibitor (100 µl inhibitor for 20 ml lysis buffer)
- Homogenize tissues using microprobe, polytron setting of “7”, 30 seconds (10 sec each time) on ice
- Transfer to 2.0 ml microcentrifuge tubes
- Centrifuge at 10,000 Xg for 10 min at 4°C
- Transfer supernatant (the total cell lysate) to a new tube
- If necessary, centrifuge supernatant again
- Take 30 µl lysated sample for protein assay
  - Save lysis buffer for blank control of protein assay
  - normally diluting 1:10 lysated sample for protein assay
  - dilutions are performed (25 µL, 50 µL, and 100 µL)
- Samples can been stored –80°C
- Boil samples at 95°C for 5 min before loading on the gel
Western Immunoblotting--Preparation of Samples:

Recipe of Laysis Buffer and Protease Inhibitor from Mitchell Lab:

Lysis Buffer:
Radioimmuno-precipitation Assay Buffer – RIFA Buffer
20 mM Tris pH 7.5
150 mM NaCl
1% Nonidet P-40
0.5% Sodium Deoxycholate
1 mM EDTA
0.1% SDS

100 ml Lysis Buffer (RIFA buffer)
• 0.24 g Tris to 75 mL distilled H2O
• add 0.877 g NaCl
• stir
• adjust pH to 7.5 with HCl
• add 10 mL 10% Nonidet P-40
• add 5 mL 10% Na-deoxycholate stock*
• stir until solution is clear
• add 1 mL 100 mM EDTA**
• add 1 mL 10% SDS***
• adjust solution to 100 mL
• store buffer at 2-8°C until ready to use

* 10% Na-deoxycholate stock solution
5 g into 50 ml
protect from light

** 100 mM EDTA stock solution
Ethylene diamine tetraacetic acid, disodium salt dyhydrate
MW 272.2
1.86 g into 40 ml H2O
add NaOH to dissolve and adjust pH to 7.4
adjust total volume to 50 mL

*** 10% SDS stock
5 g into 50 ml H2O
Protease and Phosphatase Inhibitor:
  - Freshly adding inhibitor cocktail and sodium orthovanadate activation into lysis buffer every time

Protease inhibitor cocktail:
  - mammalian protease inhibitor cocktail ordered from Sigma

  - one ml of protease inhibitor cocktail for 20 g tissues (or 10 mL of CHO cell lysate at $10^7$ cells/ml)

  - since we use 0.1 g - 0.2 g tissue, 100 µl inhibitor cocktail into 20 ml lysis buffer for 20 samples

Homemade protease inhibitor cocktail
  - PMSF 1 mM
  - Aprotinin 0.15 units/ml
  - Lenpeptin 5 µg/mL (10 µM) (MW. 475.6)
  - Pepstatin 1 µg/mL
  - Sodium fluoride 1 mM

Note: instead of making above cocktail, ordering from Sigma

Sodium orthovanadate activation:
  - Phosphatase inhibitor

  - in 20 ml lysis buffer add 100 µl of 200 mM stock of sodium orthovanadate activation and final concentration is 1 mM

  - Sodium orthovanadate activation in 200 mM stock MW 183.9
    1. 0.37 g into 10 ml H$_2$O
    2. adjust pH to 10 (solution becomes yellow)
    3. boil solution until it turns colorless (~10 min)
    4. cool to R.T.
    5. readjust pH to 10.
    6. repeat steps 3, 4 and 5 until solution remains colorless and pH stabilizes at pH 10
    7. store activated orthovanadate as aliquot at -20°C (300 µL in each aliquot)
Western Immunoblotting--Preparation of Samples:

Recipe of Lysis Buffer and Protease Inhibitor from Olson Lab:

Lysis Buffer with Homemade Protease Inhibitor

50 ml:
- 40 ml  50 mM Tris, pH 7.5
- 320 mg  NaCl
- 200 µl  200 mM stock Na-orthovanadate
- 400 µl  0.5 M EDTA, pH 8.0
- 20 µl  leupeptin stock (10 mg/ml)
- 60 µl  Aprotinin
- 40 µl  Triton X-100

1 mM PMSF* to be added just prior to use

*make a 100Mm solution of PMSF (17.42 mg/ml) in DMSO and add 10 µl per ml lysis buffer

Lysis buffer with Protease Inhibitor Cocktail

20 ml:
- 17.42 ml  Distilled water
- 1.954 ml  Tris-HCl buffer
- 200 µl  Sucrose
- 200 µl  Triton X-100

Take mixing solution 989 µl, then add fresh 10 µl cocktail and 1 µl DTT every time

Note: this recipe has no EDTA
Western Immunoblotting--Preparation of Samples:

Nuclear Extraction from Tissue:

- Grind tissue in liquid nitrogen
- Add 1 ml tissue homogenizing buffer to each 0.1 g tissue tube
- Homogenize using microprobe, polytron setting of “5”, 3 times (keep sample on ice)
- Centrifuge at 3000 rpm for 20 min at 4 °C

Cytosolic Fraction:

- Transfer supernatant to a new tube
- Ultracentrifuge at 40,000 rpm for 1 h
- Aliquot supernatant and store at –70 °C

Nuclear Extraction:

- Wash Pellet twice with homogenizing buffer
- Add nuclear extract buffer in ½ the volume of the pellet size
- Disperse gently by mixing 1 h on ice
- Centrifuge at 14,000 rpm for 20 min
- Dialyze supernatant against 50 volume of dialysis buffer for 2 h at 4 °C, change dialysis buffer after 1 h
- Centrifuge for 20 min at 14,000 rpm
- Aliquot supernatant and store at –70 °C

Tissue Homogenizing Buffer:

- 10 mM HEPES
- 1.5 mM MgCl₂
- 10 mM KCl
- 0.3 mM sucrose
- 0.1 mM EGTA
- pH 7.9
To each 50 ml add following:

<table>
<thead>
<tr>
<th>Add</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>3.8 mg</td>
</tr>
<tr>
<td>PMSF</td>
<td>125 µl</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>250 µl</td>
</tr>
<tr>
<td>Pepstain</td>
<td>50 µl</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>25 µl</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>845 µl</td>
</tr>
<tr>
<td>Sodium fluoride</td>
<td>250 µl</td>
</tr>
<tr>
<td>Noidet P-40</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>

**Nuclear Extract Buffer:**
- 20 mM HEPES
- 420 mM NaCl
- 1.5 mM MgCl₂
- 0.1 mM EGTA
- 0.2 mM EDTA
- 25 % glycerol
- pH to 7.9

To each 10 ml nuclear extract buffer add follow:

<table>
<thead>
<tr>
<th>Add</th>
<th>Final</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>0.8 mg</td>
</tr>
<tr>
<td>PMSF</td>
<td>25 µl</td>
</tr>
<tr>
<td>Sodium orthovanadate</td>
<td>50 µl</td>
</tr>
<tr>
<td>Pepstain</td>
<td>10 µl</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>5 µl</td>
</tr>
<tr>
<td>Aprotinin</td>
<td>52 µl</td>
</tr>
<tr>
<td>Spermidine</td>
<td>66.7 µl</td>
</tr>
<tr>
<td>(stock 75 mM)</td>
<td></td>
</tr>
</tbody>
</table>

**Dialysis Buffer:**
- 20 mM HEPES
- 100 mM KCl
- 0.2 mM EDTA
- 0.5 mM DTT
- 0.5 mM PMSF
- 20 % glycerol
- pH 7.9
Western Immunoblotting--Preparation of Samples:

Nuclear Extraction from Culture Cells:

- 0.5 – 1 x 10^6 cells (1 T75 flask)
- wash 3 x with ice cold PBS
- scrape cells with 1 ml PBS into 1.5 ml centrifuge tube
- Spin 3 min at 1500 xg or 5200 rpm using micro-centrifuge
- Resuspend pellet in 400 µl (about 3 x pellet cell volume) Buffer A
- Mix gently with pipet tip
- Let cells swell on ice for 15 min
- Add 25 µl 10% Noidet NP-40
- Vortex 10 sec vigorously
- Spin 30 sec at 14,000 rpm
- Cytoplasmic Fraction:
  - Transfer supernatant into a new tube
  - Store at -80 C
- Nuclear Fraction:
  - Resuspend pellet into 50 µl (about ½ pellet nuclear volume) ice cold Buffer C
  - Vigorously rock samples for 15 min at 4 C
  - Spin nuclear lysate 5 min at 14,000 rpm
  - Save supernatant at –80 C

Buffer A:

10 mM HEPES pH 7.9
10 mM KCl
0.1 mM EDTA
0.1 mM EGTA
1 mM DTT
0.5 mM PMSF
100 ml:
- 0.26 g HEPES
- 0.07 g KCl
- 3.7 mg EDTA
- 3.8 mg EGTA
- 15 mg DTT, add freshly
- 250 µl of 200mM PMSF stock, add freshly

Buffer C:
- 20 mM HEPES, pH 7.9
- 0.4 M NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM DTT, add freshly
- 1 mM PMSF, add freshly

100 ml:
- 0.52 g HEPES, pH 7.9
- 2.34 g NaCl
- 37 mg EDTA
- 38 mg EGTA
- 1.5 mg DTT, add freshly
- 0.5 ml of 200 mM PMSF stock, add freshly

PMSF stock:
- 200 mM
- MW: 174.2
- 0.174 g in 5 ml isopropanol