Western Blot

Antibodies:

1. Goat Anti-Caspase-3 (CPP32) Antibody, R&D systems (cat #AF-605-NA), 0.5 ug/ml
2. Goat Anti-human LAP (TGF-b1) Antibody, R&D Systems (cat #AF-246-NA), 0.1-0.2 ug/ml
3. Rabbit Anti Mouse Tropoelastin, Elastin Products Company (cat #PR385), 1:1000
4. mAb anti-Smad2 (L16D3), Cell Signaling (cat #3103), 1:1000
5. Rabbit anti-Smad3, Cell Signaling (cat #9513), 1:1000
6. Rabbit anti-Smad2/3, Cell Signaling (cat# 3102), 1:1000
7. Rabbit anti-Phospho-Smad2, Cell Signaling (cat # 3101), 1:1000
8. Mouse anti Phospho Smad2, Cell Signaling (cat# 3108), 1:1000
9. Rabbit anti-Phospho-Smad3/Smad1, Cell Signaling (cat # 9514), 1:1000
11. Rabbit anti-HPRT, Santa Cruz (sc-20975), 1:1000

2nd Antibodies:

1. Horseradish peroxidase Linked Sheep anti-mouse IgG (Amersham) 1:3000
2. Horseradish peroxidase Linked Donkey anti-rabbit IgG (Amersham) 1:3000

WB:

1. Transfer the electrophoresed proteins to nitrocellulose membrane (Schleicher & Schuell).
2. Block membrane in 5% fat-free dry milk-TBST (TBS + 0.1%Tween 20), 1 hour RT.
3. Wash membrane with TBST 3 times for 5 minutes.
4. Incubate membrane in primary antibody diluted in TBST-5% milk, 4°C overnight.
5. Wash membrane in TBST 3 times for 10 minutes.
6. Incubate in secondary antibody, 1 hour at room temp.
7. Wash in TBST 3 times 10 minutes.
8. Detection: ECL (Pierce, # 32106).

**Note 1:** Ab39 tends to give high background in WB. If it happens, wash membrane with TBS + 1% Triton X-100.

**Note 2:** Blocking membrane and dilute antibody in 5% BSA-TBST instead of in 5% dry milk may improve signal to noise ratio.

**Note 3:** TBS: 100mM Tris, 150 mM NaCl. pH 7.6

**Tissue Extract for WB:**

<table>
<thead>
<tr>
<th><strong>Tissue Lysis Buffer:</strong></th>
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<tbody>
<tr>
<td>10 mM HEPES pH 7.9</td>
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<tr>
<td>10 mM KCl</td>
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<tr>
<td>0.1 mM EDTA</td>
</tr>
<tr>
<td>1% TritonX-100,</td>
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<tr>
<td>1 mM glycerophosphate</td>
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<tr>
<td>2.5 mM sodiupyrophosphate,</td>
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<tr>
<td>1 mM sodium orthovanadate</td>
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</table>

1. Place mouse tissue in liquid nitrogen and grind thoroughly with a mortar and pestle.
2. Decant tissue powder and liquid nitrogen into a microcentrifuge tube. Allow the liquid nitrogen to evaporate.
3. Add appropriate volume of lysis buffer (see box) containing protease inhibitor cocktail (Roche).
4. Resuspend the tissue powder in lysis buffer well, incubate on ice for 10 minutes,
5. Homogenize by passing the lysate at least 5 times through a 22-gauge needle fitted to a syringe.
6. Centrifuge lysate for 10 minutes at full speed in a microcentrifuge at 4°C.
7. Collect supernatant and determine protein concentration by using BCA kit (Pierce). Load 50-100 ug lysate per lane in SDS-PAGE.
IHC on Parafin Sections
(Branka Dabovic, dabovb01@med.nyu.edu)

Antibodies:

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Antibody Description</th>
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<tbody>
<tr>
<td></td>
<td>Rabbit anti-Phospho-Smad2, Cell Signaling (# 3101), 1:100</td>
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<tr>
<td>B</td>
<td>Rabbit anti-Phospho-Smad1/5/8, Cell Signaling (# 9511), 1:200</td>
</tr>
<tr>
<td>B</td>
<td>Rabbit anti-Phospho-Histone H3, Cell Signaling (# 9514), 1:200</td>
</tr>
<tr>
<td>A</td>
<td>Rabbit anti-Cleaved Caspase-3, Cell Signaling (# 9664), 1:200</td>
</tr>
<tr>
<td>A</td>
<td>Rabbit anti Ki67, Novacostra (NCL-Ki67p), 1:1000-1:2000</td>
</tr>
<tr>
<td>A</td>
<td>Rabbit Anti Mouse Tropoelastin, Elastin Products Company (#PR385), 1:1000</td>
</tr>
<tr>
<td>C</td>
<td>Mouse monoclonal anti aSMA, clone 1A4 (Sigma, # A-2547), 1:100-200</td>
</tr>
<tr>
<td>D</td>
<td>Rabbit anti-proSP-C, 1:3000 (Chemicon, #AB3428) 1: 500</td>
</tr>
<tr>
<td>A</td>
<td>Rabbit anti-SA100A4 (Fsp-1), 1:300 (AnaSpec, # 53839)</td>
</tr>
<tr>
<td>A</td>
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</table>
Protocols:

Protocol A:

1) Incubate paraffin imbedded and sectioned tissue slides @50°C for about 20 min.

2) 2X Xylene/ 10’

3) 2X 100% EtOH/3’

4) 2X 95% EtOH/3’

5) 70% EtOH/3’

6) Wash in running water 10’

7) Rinse in dH₂O

8) Antigen retrieval by steaming:
   Put the slides in the white plastic Coplin jars with pre-warmed Retrieval Buffer (see the box).
   Steam the slides for 20’.
   Turn the steamer OFF and leave the slides inside the steamer to cool down for 10’.
   Take the slides out of the steamer and leave them to cool down @RT for 20’.

9) Wash 3 X 5’ with dH₂O

10) 1% H₂O₂/10’ @RT

11) Wash 2 X 5’ with dH₂O

12) TBST/5’

13) Blocking in 5% goat serum/TBST, at least 1h/RT/humid chamber
   Use ~200µl of blocking sol/slide
   Goat serum: Vector Normal Goat Serum #S-1000

14) 1st Ab in 5% goat serum TBST
   ON at 4°C in humid chamber

15) 3 washes with TBST (1x10’, 2x5’)

16) 2nd Ab: Biotinilated Anti-Rabbit IgG (H+L), made in goat, Vector;
    dilution 1:200 in 5%goat serum/TBST
    2h/RT/humid chamber

17) Wash in TBST, 10’ @ RT

18) Wash 2 X 5’ in TBS

19) ABC Vector: Vectastain kit #PK6100 2h @ RT in humid chamber
20) TBST/10'

21) Wash 2X 5' in TBS

22) Detection: DAB substrate kit for peroxidase; Vector #SK-4100

| H2O----------------------5ml |
| Buffer Stock Sol--------100µl |
| DAB Stock Sol----------50µl |
| Hydrogen Peroxide-----25µl |

Incubate tissue sections with the substrate @ RT until suitable staining develops (1'-10').

23) Wash with TBS 2-5'

24) Counterstain with hematoxylin (few seconds)
   Hematoxylin QS: Vector, #H-3404, make 50% dilution in water

25) Wash in TBS 5'

26) Mount in aqueous mounting medium (Crystal/Mount: Biomega #M02)

**Protocol B:**

**Use R&D Cell and Tissue Staining Kit (CTS005)**

1. Incubate paraffin imbedded and sectioned tissue slides @50°C for about 20 min.
2. 2 10' in Xylene
3. 2 X 3 in 100% EtOH
4. 2 X 3’ in 95% EtOH
5. 3’ in 70% EtOH
6. Wash 10’ in running water
7. Rinse in dH2O
8. Antigen retrieval by steaming:
   Put the slides in the white plastic jars with 0.01M Sodium Citrate (pH 6.0).
   Cook the slides in microwave oven: 1’ at high (p10) and 3 x 3’ at low (P1);
   cool down @RT for 20’.
9. Wash 3 X 5’ with PBS
10. Draw a circle around tissue with Pap Pen
11. Apply enough 3% H₂O₂ to cover tissue and incubate 10’ @ RT
12. Rinse with PBS and wash 5 ‘ in PBS
13. Serum Blocking 15’

14. Drain slides and carefully aspirate excess Blocking reagent. **Do not rinse with buffer.**

15. Avidin Blocking 15’
16. Rinse with PBS
17. Biotin Blocking 15’
18. Rinse with PBS

19. Apply primary AB diluted min PBS and incubate ON @ 4°C.
20. Wash 3 x 15’ in PBS
21. Incubate with 2nd Ab, 30’ @ RT.
22. Wash 3 x 15’ in PBS
23. Incubate with HSS-HRP, 30’ @ RT
24. Wash 2 x 5’ in PBS

25. DAB staining solution: 1ml DAB substrate + 1 drop of DAB chromogen, mix well.
26. Stain for 2-10’.
27. Wash 5’ in PBS
28. Counterstain with hematoxylin (10 seconds)
29. Wash in PBS for 5’

30. Mount in Cristal/Mount (Biomed #M02)
Protocol C:

Biotinilated Tyramide Amplification

1. Incubate paraffin imbedded and sectioned tissue slides @50°C for about 20 min.
2. 2 X 10’ Xylene
3. 2 X 3’ 100% EtOH
4. 2 X 3’ 95% EtOH
5. 3’ 70% EtOH
6. Wash in running water for 10’
7. Rinse in dH2O

14. Incubate in 1% H2O2 for 10’ @ RT
15. Wash 2 X 5’ with dH2O

16. Wash 5’ in TBST

17. Block sections in 5% goat serum/TBST (or Perkin Elmer TSA blocking solution) for at least 1h at RT in humid chamber
   Goat serum: Vector Normal Goat Serum #S-1000

18. 1st Ab in 5% goat serum/TBST (or Perkin Elmer TSA blocking solution)
    ON 4°C humid chamber

19. 3 washes with TBST 1 X 10’ + 2 X 5’.

20. Incubate the slides with HRP-conjugated 2nd Ab, 1-2 h @ RT. (For rabbit Ab-s we use Jackson Imm Res. Ab # 111-035-006, 1:200)

21. Wash in TBST for 10’ at RT
22. Wash 2 X 5’ in TBS

23. Incubate the slides with Bio-Tyramide working solution (2µg/ml in TBS) 3-10 min. (3 min should be enough)

24. Wash 3 x 5 min
Then:

**DAB based detection:**

25a. ABC Vector: Vectastain kit #PK6100 2h @ RT in humid chamber

26a. TBST/10’

27a. Wash 2x 5’ in TBS

28a. Detection: DAB substrate kit for peroxidase; Vector #SK4100

29a. Incubate tissue sections with the substrate @ RT until suitable staining develops (1’–10’).

30a. Wash with TBS 2-5'

31a. Counterstain with hematoxylin (few seconds)

   Hematoxylin QS: Vector, #H-3404, make 50% dilution in water

32a. Wash in TBS 5’

33a. Mount in aqueous mounting medium (Crystal/Mount: Biomeda #M02)

Or

**Fluorescent staining:**

25b. Incubate with Streptavidin – Alexa 568 1:1000 in TBS (or PBS), 30 min @ RT

26b. Wash 3 x 5 min in TBS

27b. Counterstain with DAPI 10 µg/ml for 5 min

28b. Wash 3 x 5 min in TBS

29b. Mount with Fluoromount-G: SouthernBiotech #0100-01.
Protocol D. Use vector M.O.M. Kit, peroxides (# PK-2200)

Follow protocol A up to step 11. Than follow M.O.M. Staining procedure from step 4 to the end.

Modifications of MOM protocol:
1. All washes, but in step 8, were increased to 5 min.
2. Incubation with 1st Ab (step 10) was increased to 1h.
3. Incubation with 2nd Ab (step 12) was increased to 30 min.
4. Steps 13 and 15. : wash 3 X 5 min.
5. Prepare ABC and DAB staining solutions as in protocol A.