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Immunohistochemistry (without pretreatment)

1. Deparaffinization
2. Xylen substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H$_2$O$_2$ in Methanol, room temp. 30 min.
4. Hydration: 
   80% Ethanol → 70% Ethanol → 60% Ethanol → rinse by running water, 1 min.
5. Rinse by TBS-T
6. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum), room temp. 30 min.
7. Wash by TBS-T
8. Primary antibody incubation: 4°C overnight
9. Wash by TBS-T, 5 min. x 3
10. Secondary antibody incubation: room temp. 30 min.
    for example, Anti-rabbit IgG goat antibody-Biotin
11. Wash by TBS-T, 5 min. x 3
12. Staining system: room temp. 30 min.
    (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
13. Wash by TBS-T, 5 min. x 3
14. Chromogenic reaction: room temp. 1-10 min.
    (DAB, ” DOJINDO 349-00903” 30 mg, 30 % H$_2$O$_2$ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
15. Rinse by running water, 3 min.
16. Counter staining
Immunohistochemistry (Microwave pretreatment)

1. Deparaffinization
2. Xylen substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H₂O₂ in Methanol, room temp. 30 min.
4. Hydration:
   80% Ethanol → 70% Ethanol → 60% Ethanol → rinse by running water, 1 min.
5. Microwave treatment*: 90 °C, 10 min (10 mM Citrate buffer, pH 6.0)
6. Cool down (leave it lay).
7. Rinse by running water, 3 min.
8. Rinse by TBS-T
9. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum), room temp. 30 min.
10. Wash by TBS-T
11. Primary antibody incubation: 4°C overnight
12. Wash by TBS-T, 5 min. x 3
13. Secondary antibody incubation: room temp. 30 min.
   for example,
   Anti-rabbit IgG goat antibody-Biotin
   Anti-mouse IgG goat antibody-Biotin
14. Wash by TBS-T, 5 min. x 3
15. Staining system: room temp. 30 min.
   (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
16. Wash by TBS-T, 5 min. x 3
17. Chromogenic reaction: room temp. 1-10 min.
   (DAB, "DOJINDO 349-00903" 30 mg, 30 % H₂O₂ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
18. Rinse by running water, 3 min.
19. Counter staining

*Microwave treatment (case of using a household kitchen microwave)
1) Put 500mL of buffer into a 500mL beaker, and then soak the whole basket with tissue sections inside in the beaker.
2) Heat for 10 minutes (500 W) after boiling in a kitchen microwave.
   Note: Cover the beaker with a plastic wrap loosely to avoid evaporation of the buffer.

Preparation of 10 mM Citrate buffer, pH 6.0
1) Solve 2.1 g of citric acid (C₆H₈(OH)(COOH)₃/H₂O=210.14) in 900 mL deionized water.
2) Adjust the pH to 6.0 with sodium hydroxide solution (add about 13 mL as 2M-NaOH).
3) Fill deionized water to a volume of 1,000 mL.
**Immunohistochemistry (Autoclave pretreatment)**

1. Deparaffinization
2. Xylen substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H$_2$O$_2$ in Methanol, room temp. 30 min.
4. Hydration:
   
   80% Ethanol → 70% Ethanol → 60% Ethanol → rinse by running water, 1 min.
5. Autoclave treatment*: 110 °C, 10 min (10 mM Citrate buffer, pH 6.0)
6. Cool down (leave it lay).
7. Rinse by running water, 3 min.
8. Rinse by TBS-T
9. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum), room temp. 30 min.
10. Wash by TBS-T
11. Primary antibody incubation: 4°C overnight
12. Wash by TBS-T, 5 min. x 3
13. Secondary antibody incubation: room temp. 30 min.
   
   for example,
   
   Anti-rabbit IgG goat antibody-Biotin
   
   Anti-mouse IgG goat antibody-Biotin
14. Wash by TBS-T, 5 min. x 3
15. Staining system: room temp. 30 min.
   
   (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
16. Wash by TBS-T, 5 min. x 3
17. Chromogenic reaction: room temp. 1-10 min.
   
   (DAB, ” DOJINDO 349-00903” 30 mg, 30 % H$_2$O$_2$ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
18. Rinse by running water, 3 min.
19. Counter staining

*Autoclave treatment

1) Put 500mL of buffer into a 500mL beaker, and then soak the whole basket with tissue sections inside in the beaker.
2) Heat for 10 minutes (110 °C) by autovlave.

Preparation of 10 mM Citrate buffer, pH 6.0

1) Solve 2.1 g of citric acid (C$_3$H$_4$(OH)(COOH)/H$_2$O=210.14) in 900 mL deionized water.
2) Adjust the pH to 6.0 with sodium hydroxide solution (add about 13 mL as 2M-NaOH).
3) Fill deionized water to a volume of 1,000 mL.
Immunohistochemistry (Trypsin pretreatment)

1. Deparaffinization
2. Xylen substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H₂O₂ in Methanol, room temp. 30 min.
4. Hydration:
   80% Ethanol → 70% Ethanol → 60% Ethanol → rinse by running water, 1 min.
5. Rinse by TBS-T
6. Trypsin pretreatment: (0.1 %), room temp. 30 min.
   *Condition setting by each laboratory is recommended as reactions may vary depending on the type of tissue or condition of fixation.
7. Rinse by TBS-T
8. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum), room temp. 30 min.
9. Wash by TBS-T
10. Primary antibody incubation: 4°C overnight
11. Wash by TBS-T, 5 min. x 3
12. Secondary antibody incubation: room temp. 30 min.
   for example,
   Anti-rabbit IgG goat antibody-Biotin
   Anti-mouse IgG goat antibody-Biotin
13. Wash by TBS-T, 5 min. x 3
   (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
15. Wash by TBS-T, 5 min. x 3
16. Chromogenic reaction: room temp. 1-10 min.
   (DAB, "DOJINDO 349-00903" 30 mg, 30 % H₂O₂ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
17. Rinse by running water, 3 min.
18. Counter staining
Immunohistochemistry (Formic acid pretreatment)

1. Deparaffinization
2. Xylen substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H₂O₂ in Methanol, room temp. 30 min.
4. Hydration:
   80% Ethanol → 70% Ethanol → 60% Ethanol → rinse by running water, 1 min.
5. Formic acid treatment: more than 99 % formic acid, room temp. 5 min.
   (acceptable concentration is over 70 %)
6. Rinse by running water, 3 min.
7. Rinse by TBS-T
8. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum),
   room temp. 30 min.
9. Wash by TBS-T
10. Primary antibody incubation: 4°C overnight
11. Wash by TBS-T, 5 min. x 3
12. Secondary antibody incubation: room temp. 30 min.
   for example,
   Anti-rabbit IgG goat antibody-Biotin
   Anti-mouse IgG goat antibody-Biotin
13. Wash by TBS-T, 5 min. x 3
   (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
15. Wash by TBS-T, 5 min. x 3
16. Chromogenic reaction: room temp. 1-10 min.
   (DAB, ”DOJINDO 349-00903” 30 mg, 30 % H₂O₂ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
17. Rinse by running water, 3 min.
18. Counter staining
Immunohistochemistry
(Microwave or Autoclave treatment after Formic acid pretreatment)

1. Deparaffinization
2. Xylene substitution for Ethanol
3. Inactivation of endogenous peroxidases: 0.3% H$_2$O$_2$ in Methanol, room temp. 30 min.
4. Hydration: 80% Ethanol $\rightarrow$ 70% Ethanol $\rightarrow$ 60% Ethanol $\rightarrow$ rinse by running water, 1 min.
5. Formic acid treatment: more than 99% formic acid, room temp. 5 min.
   (acceptable concentration is over 70%)
6. Rinse by running water, 3 min.
7. Microwave treatment* or
   Autoclave treatment: 110 °C, 10 min (10 mM Citrate buffer, pH 6.0)
8. Cool down (leave it lay).
9. Rinse by running water, 3 min.
10. Rinse by TBS-T
11. Blocking: 5% normal serum of secondary antibody animal (e.g. goat whole serum),
    room temp. 30 min.
12. Wash by TBS-T
13. Primary antibody incubation: 4°C overnight
14. Wash by TBS-T, 5 min. x 3
15. Secondary antibody incubation: room temp. 30 min.
    for example,
    Anti-rabbit IgG goat antibody-Biotin
    Anti-mouse IgG goat antibody-Biotin
16. Wash by TBS-T, 5 min. x 3
17. Staining system: room temp. 30 min.
    (Vectastatin ABC Kit, PEROXIDASE STANDARD PK-4000)
18. Wash by TBS-T, 5 min. x 3
19. Chromogenic reaction: room temp. 1-10 min.
    (DAB, "DOJINDO 349-00903" 30 mg, 30 % H$_2$O$_2$ 25 μL/50 mM Tris-HCl, pH 7.6, 150 mL)
20. Rinse by running water, 3 min.
21. Counter staining

*Microwave treatment (case of using a household kitchen microwave)
1) Put 500mL of buffer into a 500mL beaker, and then soak the whole basket with tissue sections inside in the beaker.
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   Note: Cover the beaker with a plastic wrap loosely to avoid evaporation of the buffer.

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2) Adjust the pH to 6.0 with sodium hydroxide solution (add about 13 mL as 2M-NaOH).
3) Fill deionized water to a volume of 1,000 mL.
Antibody Absorption Test by Antigen Peptide

1) Add antibody solution and peptide solution into antibody dilution buffer (1 % BSA in PBS).
   The ratio of antibody and peptide is 1 mol : 20 mol.
   In this case, use deionized water instead of peptides solution as control.

2) Incubation with rotating for overnight at 4°C.
   Treat the control in parallel.

3) Use the treated solutions as primary antibody.

Calculation for step 1).

For example  #18134  HGF-beta (H495) Rabbit IgG

Molecular weight of Antigen Peptide for HGF-beta (H495) is 1,847.15.
And we assume the molecular weight of our antibody product is about 150,000

Rate of
Antibody : Peptide
= 1 mol : 20 mol
= 150,000 : 1,847 x 20
= 1 g : 0.24 g

In the case of ;
Starting concentration of antibody solution is 100 μg/mL.
Starting concentration of peptides solution is 100 μg/mL.
Concentration of antibody for use in IHC or W.B. is 5 μg/mL.
If you would like to make 1 mL of solution, please confect them as below.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody solution (100 μg/mL)</th>
<th>Peptides solution (100 μg/mL)</th>
<th>1 % BSA in PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide (+)</td>
<td>50 μL</td>
<td>12 μL</td>
<td>938 μL</td>
</tr>
<tr>
<td>Peptide (-)</td>
<td>50 μL</td>
<td>-</td>
<td>950 μL</td>
</tr>
</tbody>
</table>
Western Blotting

Reagents

- **2x Sample buffer**
  125mM Tris-HCl (pH6.8), 4% SDS, 20% Glycerol, 10% 2-Mercaptoethanol, 0.02% BPB

- **HRP conjugated secondary antibody**
  Anti-Rabbit IgG (H+L) Goat IgG Fab’ HRP (IBL, #17502) or
  Anti-Mouse IgG (H+L) Goat IgG Fab’ HRP (IBL, #17601)

- **Blocking solution**
  3% milk, 1% BSA, 0.05% NaN₃ / PBS

- **Washing solution**
  0.05% Tween20/PBS

- **ECL Western Blotting Detection Reagent**
  GE Healthcare, # RPN2106

Procedure

1. PAGE: Apply 10 - 20μL of prepared sample to polyacrylamide gel (7-12 %).
2. Electrophoresis
4. Blocking the membrane with blocking solution: 2hrs. at 37°C
5. Wash with washing solution, 5 min. x 3 times
6. Primary antibody: 2hrs. at 37°C or overnight at 4°C
7. Wash with washing solution, 5 min. x 3 times
8. Second antibody: 1hr. at 37°C
9. Wash with washing solution, 5 min. x 3 times
10. Detection with ECL

Example of sample preparation

**Cell lysate**

1) Wash the cultured cells with PBS and trypsin treatment as necessary.
2) Wash with PBS after cessation of trypsin action and count the number of cells.
3) Suspend the cells in 2x Sample buffer (1 - 5 x 10⁵ cells/10μL).
4) Sonication
5) Boiling (heat block), 3min.
6) Centrifugation, 14,000 rpm. 3min. at 4°C
7) Use the supernatant.

**Cell culture medium**

Use the supernatant
Immuno-precipitation

Reagent

1. **TNE buffer:**
   
   10 mM Tris-HCl (pH7.8), 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 10 μg/mL aprotinin

2. **Protein G-Sepharose 4 Fast Flow (GE Healthcare #17-0618-01):**
   
   Be washed by TNE buffer

Procedure

1. **Add Protein G-Sepharose to prepared sample (e.g. extraction supernatant):**
   
   : 50 μL/1 mL sample

2. **Rotating incubation:** 4°C overnight

3. **Centrifugation:** 4 °C 14,000 rpm 20 min.

4. **Add antibody to supernatant:** about 3 μg/100 – 400 μL

5. **Rotating incubation:** 4°C 1 hr

6. **Add Protein G-Sepharose to the solution:** 20 μL/100 – 400 μL

7. **Rotating incubation:** 4°C 1 hr

8. **Wash the pellet by TNE buffer, 4 °C 5,000 rpm 1 min x 5 times**

9. **Immunoprecipitate**

10. **Use for western blot or other experiment**