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Immunohistochemistry (without pretreatment)

1. **Deparaffinization**
2. **Xylen substitution for Ethanol**
3. **Inactivation of endogenous peroxidases: 0.3% H_2O_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
    Anti-mouse 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_2O_2 25 µL/50 mM Tris-HCl, pH 7.6, 150 mL)
15. **Rinse by running water, 3 min.**
16. **Counter staining**

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Immunohistochemistry (Microwave pretreatment)

1. **Deparaffinization**
2. **Xylen substitution for Ethanol**
3. **Inactivation of endogenous peroxidases: 0.3% H_2O_2 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**
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    (DAB, ” DOJINDO 349-00903” 30 mg, 30 % H_2O_2 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 ($C_3H_4(OH)(COOH)_3/H_2O$=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_2O_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**
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    (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_2O_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 ($C_3H_4(OH)(COOH)_3/H_2O$=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.

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Immunohistochemistry (Trypsin pretreatment)

1. **Deparaffinization**
2. **Xylen substitution for Ethanol**
3. **Inactivation of endogenous peroxidases: 0.3% H_2O_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. **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,
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13. **Wash by TBS-T, 5 min. x 3**
14. **Staining system: room temp. 30 min.**
    (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 % H_2O_2 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% H_2O_2 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**
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13. **Wash by TBS-T, 5 min. x 3**
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    (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 % H_2O_2 25 µL/50 mM Tris-HCl, pH 7.6, 150 mL)
17. **Rinse by running water, 3 min.**
18. **Counter staining**

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Immunohistochemistry
(Microwave or Autoclave treatment after Formic acid pretreatment)

1. **Deparaffinization**
2. **Xylen substitution for Ethanol**
3. **Inactivation of endogenous peroxidases: 0.3% H_2O_2 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. **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.**
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Preparation of 10 mM Citrate buffer, pH 6.0

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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.

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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.

	Antibody solution (100 µg/mL)	Peptides solution (100 µg/mL)	1 % BSA in PBS
Peptide (+)	50 µL	12 µL	938 µL
Peptide (-)	50 µL	-	950 µL

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% NaN_3 / 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**
3. **Blotting: Transfer to a nylon membrane.**
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 10^5 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**