Immunohistochemistry (IHC-P) Protocol

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections. Immunohistochemical staining is accomplished to recognize the target protein with antibody which specifically binds to the protein of interest in the tissue section. IHC-P refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned.

1. Reagents

Xylene

Store at room temperature

100% Ethanol

Store at room temperature

95% Ethanol

95 ml 100% Ethanol
5 ml ddH_2O
Store at room temperature

90% Ethanol

90 ml 100% Ethanol
10 ml ddH_2O
Store at room temperature

80% Ethanol

80 ml 100% Ethanol
20 ml ddH_2O
Store at room temperature

70% Ethanol

70 ml 100% Ethanol
30 ml ddH_2O
Store at room temperature

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Sodium Citrate Buffer

2.94 g Sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$)

0.44g $C_6H_8O_7 \cdot 2H2O$

1000 ml ddH_2O

Adjust to pH 6.0

Store at room temperature

Hydrogen Peroxide Buffer

10 ml 30%H_2O_2

90 ml ddH_2O

Store at room temperature

PBS Buffer

8.5 g NaCl

1.4 g Na_2HPO_4

0.2 g NaH_2PO_4

1000 ml ddH_2O

Adjust to pH 7.4

Store at 4 °C

Washing Buffer

0.5 ml Tween 20

1000 ml PBS Buffer

Store at 4 °C

Antibody Dilution Buffer

3 g BSA

100 ml PBS Buffer

Store at 4 °C

Normal Blocking Solution

4.5 ml PBS Buffer

0.5 ml Normal goat serum

Store at 4 °C

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Avidin/Biotin Blocking Solution

2 ml Egg white

8 ml PBS Buffer

Store at 4 °C

DAB Reagent

Hematoxylin Reagent

2. Procedure

2.1 Deparaffinization/Rehydration

a. Immerse sections twice with *Xylene* for 10 minutes each time.

b. Immerse sections twice with *100% Ethanol* for 10 minutes each time.

c. Immerse sections once with *95% Ethanol* for 5 minutes.

d. Immerse sections once with *90% Ethanol* for 5 minutes.

e. Immerse sections once with *80% Ethanol* for 5 minutes.

f. Immerse sections once with *70% Ethanol* for 5 minutes.

g. Immerse sections with *PBS Buffer* for 5 minutes and immerse the sections in ddH_2O until antigen retrieval.

Note: Do not allow sections to dry at any time until ready to perform antigen retrieval. Drying will cause non-specific antibody binding and therefore high background staining.

2.2. Endogenous peroxidase inhibition

a. Immerse sections with *Hydrogen Peroxide Buffer* for 10 minutes at room temperature.

b. Wash sections with ddH_2O twice for 5 minutes each time.

2.3. Antigen retrieval

a. Immerse section in *Sodium Citrate Buffer* then maintain at 100 °C for 10 minutes.

b. Cool section on bench top for 30 minutes.

c. Wash sections with ddH_2O twice for 5 minutes each time.

2.4. Normal blocking

a. Immerse each section with 100-400 µl of *Normal Blocking Solution* for 1 hour at room temperature.

b. Rinse the excess *Normal Blocking Solution* with a gentle stream of *PBS Buffer* from a wash buffer bottle and wash section with *Washing Buffer* for 10 minutes.

2.5 Avidin/Biotin blocking

a. Immerse each section with 100-400 µl of *Avidin/Biotin Blocking Solution* at 37 °C for 30 minutes.

b. Rinse the excess Blocking Buffer with a gentle stream of *PBS Buffer* from a wash buffer bottle and wash section with *Washing Buffer* for 10 minutes.

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2.6. Primary incubation

a. Dilute primary antibody and negative control antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer's guidance.

b. Add 100-400 µl of diluted primary antibody and negative control antibody to each section and incubate at 37 °C for 1 hour or 4 °C for 12 to 24 hours in a closed incubation chamber.

c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.

Note: The concentration of antibody should be diluted according to manufacturer's recommendations.

2.7. Biotin-labeled secondary antibody incubation

a. Dilute Biotin-labeled antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer's guidance.

b. Add 100-400 µl of diluted Biotin-labeled antibody to each section and incubate at 37 °C for 45 minutes in an incubation chamber.

c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.

2.8. Streptavidin-HRP incubation

a. Dilute Streptavidin-HRP antibody with *Antibody Dilution Buffer* at proper working concentration according to manufacturer's guidance.

b. Add 100-400 µl of diluted Streptavidin-HRP to each section and incubate at 37 °C for 30 minutes in an incubation chamber.

c. Rinse the excess antibody from the section with a gentle stream of *PBS Buffer* from a wash bottle and immerse sections in *Washing Buffer* twice for 10 minutes at room temperature.

2.9. DAB stain

a. Add 100-400 µl of *DAB Reagent* or other substrate to each section and react for 2 to 7 minutes to stain.

b. Rinse sections with *PBS buffer* to stop staining.

Note: The following step is additional but not necessary.

2.10. Hematoxylin stain

Counterstain sections in *Hematoxylin Reagent* according to manufacturer's instructions.

2.11. Dehydrate sections

a. Immerse sections with *95% Ethanol* twice for 10 seconds each time.

b. Immerse sections with *100% Ethanol* twice for 10 seconds each time.

c. Immerse sections with *Xylene*, incubating sections for 10 seconds each time.

2.12. Mount coverslips

2.13. Viewing the staining under the microscope.

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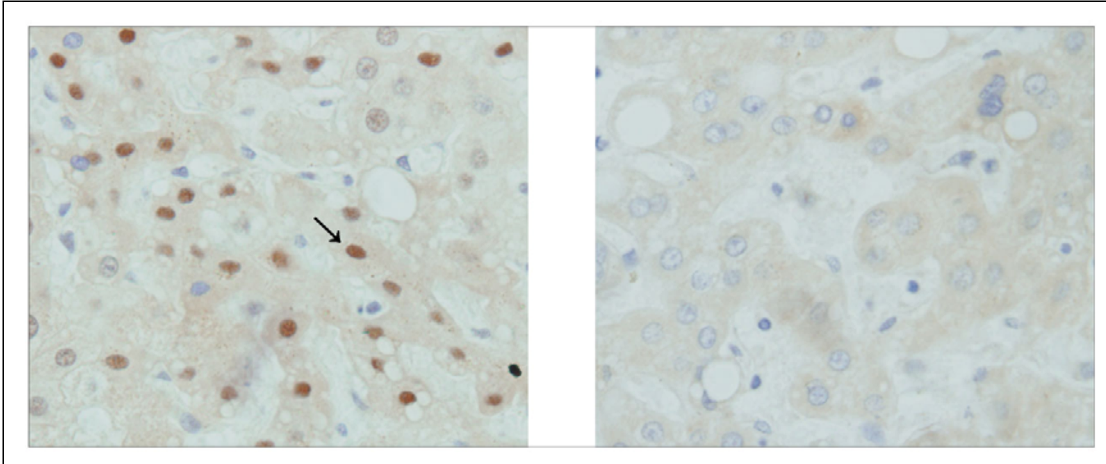
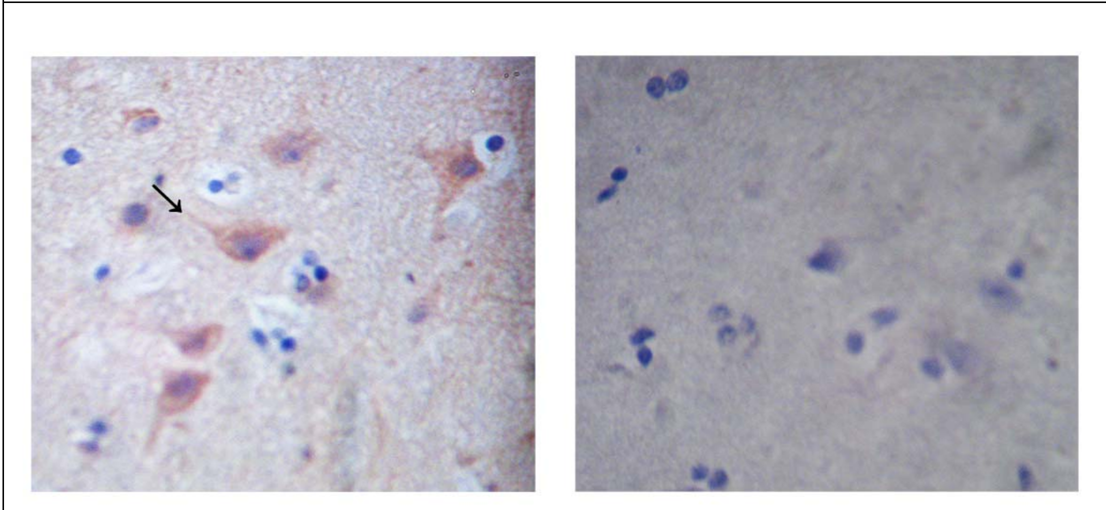
3. Immunohistochemistry examples



Immunohistochemistry analysis of human carcinoma hepatis tissue slide (Paraffin embedded) using Ki-67 Antibody, pAb, Rabbit (Left, GenScript, A01495) and Purified Rabbit IgG (Whole molecule) Control (Right, GenScript, A01008).



Immunohistochemistry analysis of human brain tissue slide (Paraffin embedded) using β-Tubulin III Antibody, pAb, Rabbit (Left, GenScript, A01203) and Purified Rabbit IgG (Whole molecule) Control (Right, GenScript, A01008).

4. Recommended Products

Name	Cat. No.
Streptavidin-HRP	M00091
Human IgG Control (Whole Molecule), Purified	A01006
Mouse IgG Control (Whole Molecule), Purified	A01007
Rabbit IgG Control (Whole Molecule), Purified	A01008
Goat IgG Control (Whole Molecule), Purified	A01009
Chicken IgY Control (Whole Molecule), Purified	A01010

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5. Troubleshooting

Problem	Possible Cause	Solution
Weak or no staining	Inadequate deparaffinization	Deparaffinize sections longer or change fresh xylene
	Inactive primary antibodies	Replace with a new batch of antibodies
	Antibody concentration is too low	Increase the concentration of primary and/or secondary antibodies
	Incompatible secondary and primary antibodies	Use secondary antibody that will interact with primary antibody
	Inactive secondary antibody	Replace with a new batch of antibody
Overstaining	Inadequate substrate incubation time	Increase the substrate incubation time
	The concentration of primary and/or secondary antibodies is too high	Reduce antibody concentration or perform a titration to determine the optimal dilution for primary and secondary antibodies
	Incubation time is too long	Reduce incubation time
	Substrate incubation time is too long	Reduce substrate incubation time
	Sections dried out	Avoid sections being dried out
High background	Inadequate washing of sections	Wash at least 3 times between steps
	Non-specific binding of primary antibodies to tissue or antibody concentration was too high	Non-specific binding may be reduced by using higher dilution of primary antibodies
	Non-specific binding of secondary antibodies to tissue	Treat tissue with normal serum from the same species as secondary antibodies.
	Secondary antibodies cross react with similar species of tissue	Use pre-adsorbed 2nd antibody, i.e. use rabbit anti-rat IgG, mouse adsorbed, on mouse tissue, or use rabbit anti-mouse IgG, rat adsorbed, on rat tissue
	Diffusion of tissue antigen due to inadequate fixation	Increase duration of postfixation

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