# IHC-P Protocol Version 2

**CJ Xia**

## Abstract

Immunohistochemistry (IHC) is a method that combines biochemical, histological and immunological techniques into a simple but powerful assay for protein detection. IHC provides valuable information as it visualizes the distribution and localization of specific cellular components within cells and in proper tissue context.

This protocol describes the steps for performing the immunohistochemistry method with paraffin-embedded tissue sections.

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## Guidelines

Tissue preparation is a key to successful IHC experiments. Since no universal tissue preparation method will be ideal for all sample and tissue types, the protocol given here is intended as a starting point from which the experimenter must optimize as needed. All conditions should be standardized in order to ensure reproducible results. Keep in mind that you must be careful not to allow tissues to dry out at any time.

## Protocol

### Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 1.**

Harvest fresh tissue and place it in a dish filled with ice-cold PBS buffer (AR0030, Boster Bio).

REAGENTS
Phosphate Buffered Saline (PBS) Powder AR0030 by Boster Bio

### Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 2.**

Wash the tissue thoroughly with PBS to remove blood. Use forceps to remove connective tissues.

### Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 3.**

Cut the tissue into slices with a thickness of 3 mm or less.

Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 4.**

Immerse the slices in 4% paraformaldehyde (AR1068, Boster Bio) at room temperature for 8 minutes.

REAGENTS

4% Paraformaldehyde (PFA) Solution in PBS AR1068 by Boster Bio

Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 5.**

Immerse the slices in 4% paraformaldehyde (pre-cool at 4°C) for 6 to 7 hours. The paraformaldehyde volume should be 20X greater than the tissue volume by weight.

Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 6.**

Wash the tissue 3X with PBS (1 minute each).

Sample Preparation - Paraformaldehyde Cooling and Dehydration

**Step 7.**

Dehydrate the tissue by immersing the tissue sequentially as follows:

- 1X into 80% ethanol (1 hour at 4°C)
- 1X into 90% ethanol (1 hour at 4°C)
- 3X into 95% ethanol (1 hour each at 4°C)
- 3X into 100% ethanol (1 hour each at 4°C)
- 3X into dimethylbenzene (0.5 hr each at room temperature)

Sample Preparation - Liquid Paraffin Section

**Step 8.**

Prepare the first portion of liquid paraffin in a suitable bath and allow the paraffin to reach and maintain at 60°C.

TEMPERATURE

60 °C Additional info:

Sample Preparation - Liquid Paraffin Section

**Step 9.**

Immerse the tissue 2X into the paraffin bath (2 hours each).

Sample Preparation - Liquid Paraffin Section

**Step 10.**

Prepare the second portion of liquid paraffin in a suitable bath and allow the paraffin to reach and maintain at 60°C.

TEMPERATURE
60 °C Additional info:

Sample Preparation - Liquid Paraffin Section
**Step 11.**

Pour the second portion of paraffin into a mold.

Sample Preparation - Liquid Paraffin Section
**Step 12.**

Quickly transport the tissue from the paraffin bath to the mold with paraffin.

Sample Preparation - Liquid Paraffin Section
**Step 13.**

Incubate the tissue at room temperature until it coagulates.

Sample Preparation - Liquid Paraffin Section
**Step 14.**

Store the tissue at 4°C.

TEMPERATURE
4 °C Additional info:

Sample Preparation - Section Slicing and Incubation
**Step 15.**

Secure the paraffin section on the slicer.

Sample Preparation - Section Slicing and Incubation
**Step 16.**

Slice one to two pieces of the section to adjust the slicer so that the section and blade are parallel

Sample Preparation - Section Slicing and Incubation
**Step 17.**

Slice the remaining section carefully with 5 µm thickness.

Sample Preparation - Section Slicing and Incubation
**Step 18.**

Incubate the sliced section in 40 to 50°C water to unfold.

Sample Preparation - Section Slicing and Incubation
**Step 19.**

Mount the tissue section onto Poly-Lysine (AR0003, Boster Bio) or APTES (AR0001, Boster Bio) coated glass slides.

REAGENTS

Poly-L-Lysine Solution, 10X, For 100-200 Slides AR0003 by Boster Bio

3-Aminopropyltriethoxysilane (APTES) 10ml 50X Concentrated AR0001 by Boster Bio

## Sample Preparation - Section Slicing and Incubation

**Step 20.**

Incubate the slides overnight at 37°C.

TEMPERATURE

37 °C Additional info:

NOTES

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This fixation procedure using paraformaldehyde and formalin fixatives may cause autofluorescence in the green spectrum. In this case, you may try fluorophores in the (i) red range or (ii) infrared range if you have an infrared detection system.

## Dewaxing/Deparaffinization

**Step 21.**

Prepare the following reagents:

- 90% dimethylbenzene
- 95% dimethylbenzene
- 100% dimethylbenzene
- 90% ethanol
- 95% ethanol
- 100% ethanol

## Dewaxing/Deparaffinization

**Step 22.**

Sequentially immerse paraffin sections into:

- 90% dimethylbenzene (for 7 minutes)
- 95% dimethylbenzene (for 7 minutes)
- 100% dimethylbenzene (for 7 minutes)
- 90% ethanol (for 7 minutes)
- 95% ethanol (for 7 minutes)
- 100% ethanol (for 7 minutes)

## Dewaxing/Deparaffinization

**Step 23.**

Wash the slides with water to remove the ethanol.

NOTES
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The process of dewaxing should be done in a fume hood at room temperature in summer. When the temperature is lower than 18°C, it is recommended to dewax at 50°C.

Inactivation
**Step 24.**

Immerse dewaxed paraffin section into the 3% $H_2O_2$ (AR1108, Boster Bio) at room temperature for 10 minutes.

REAGENTS
3% Hydrogen Peroxide (H2O2) Solution, Lab Grade AR1108 by Boster Bio

Inactivation
**Step 25.**

Wash the section 3X to 5X with distilled water (total 3 to 5 minutes).

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 26.**

Immerse the paraffin sections in citrate buffer (AR0024, Boster Bio).

REAGENTS
Citrate Buffer Powder 2L/Pack, For Heat Antigen Retrieval AR0024 by Boster Bio

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 27.**

Heat the buffer in the microwave and turn it off when the buffer has boiled.

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 28.**

Keep the boiled buffer in the microwave for 5 to 10 minutes.

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 29.**

Repeat the heating as outlined above for 1 to 2 times.

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 30.**

Cool the slides until it reaches room temperature.

Antigen Retrieval (Heat Induced Epitope Retrieval: HIER)
**Step 31.**

Wash the sections 1X to 2X with PBS.

Blocking

**Step 32.**

Add 5% BSA blocking solution or normal goat serum to the HIER treated samples.

Blocking

**Step 33.**

Incubate the samples at 37°C for 30 minutes.

TEMPERATURE

37 °C Additional info:

Blocking

**Step 34.**

Discard extra liquid (No washing required).

Primary Antibody Incubation

**Step 35.**

Dilute primary antibody with antibody diluent (AR1016, Boster Bio) to the concentration recommended by the antibody manufacturer.

REAGENTS

Antibody Dilution Buffer With BSA, Reducing Background AR1016 by Boster Bio

Primary Antibody Incubation

**Step 36.**

Add the diluted antibody to the samples and incubate overnight at 4℃ or at 37℃ for 1 hour.

Primary Antibody Incubation

**Step 37.**

Wash the samples 2X with PBS (20 minutes each).

Secondary Antibody Incubation

**Step 38.**

Dilute biotinylated secondary antibody with antibody diluent (AR1016, Boster Bio) to the concentration recommended by the antibody manufacturer.

REAGENTS

Antibody Dilution Buffer With BSA, Reducing Background AR1016 by Boster Bio

Secondary Antibody Incubation

**Step 39.**

Add the diluted antibody to the samples and incubate at 37°C for 30 minutes.

TEMPERATURE
37 °C Additional info:

Secondary Antibody Incubation

**Step 40.**

Wash the samples 2X with PBS (20 minutes each).

Staining

**Step 41.**

Add Strept-Avidin Biotin Complex (SABC) HRP-conjugated or AP-conjugated reagents to the samples.

LINK:
https://www.bosterbio.com/catalogsearch/result/?q=sabc+alkaline+phosphatase

Staining

**Step 42.**

Incubate the samples at 37°C for 30 minutes.

TEMPERATURE
37 °C Additional info:

Staining

**Step 43.**

Wash the samples 3X with PBS (20 minutes each).

Staining

**Step 44.**

Add a suitable amount of DAB reagent (AR1022, AR1025, Boster Bio) to the samples and incubate in darkness at room temperature for 10 to 30 minutes.

REAGENTS
DAB Chromogenic Substrate Reagent Kit (Yellow) AR1022 by Boster Bio
DAB Chromogenic Substrate Reagent Kit (Blue) AR1025 by Boster Bio

LINK:
https://www.bosterbio.com/catalogsearch/result/index/?applications=17&q=dab+chromogenic

Staining

**Step 45.**

Monitor the tissue staining intensity under a bright-field microscope.

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If the staining background is too high, wash the section 4X with 0.01-0.02% TWEEN 20 PBS and 2X

with pure PBS after the SABC reaction and before DAB staining. Then use DAB to stain the samples.

Staining

**Step 46.**

Wash the samples 3 to 5 times with distilled water.

Staining

**Step 47.**

Counterstain (if necessary)

- Add haematoxylin (AR0005, Boster Bio) to the sample
- Dehydrate
- Immerse the paraffin sections 2X in dimethylbenzene (7 minutes each)

REAGENTS

Hematoxylin Counterstain Solution AR0005 by Boster Bio

Staining

**Step 48.**

Check the tissue staining intensity under a bright-field microscope.