🔬 Immunohistochemistry (IHC)

DAB-Based Antibody Detection Protocol

Protocol Overview

Immunohistochemistry (IHC) is a technique that uses enzyme-labeled antibodies to detect specific proteins or antigens in tissue sections. This protocol uses the ABC (Avidin-Biotin Complex) method with DAB (3,3'-Diaminobenzidine) chromogen for brown colorimetric detection.

⏱️ Duration

Day 1: ~4 hours (+ overnight incubation)

Day 2: ~4 hours

Total: 2 days

🎯 Key Steps

1. Deparaffinization & antigen retrieval
2. Peroxide quench & blocking
3. Primary antibody (overnight)
4. Secondary antibody & ABC
5. DAB development & counterstain

💡 Important Notes:

- Prepare ABC solution at least 30 minutes before use
- Prepare DAB immediately before use - it's light-sensitive
- Monitor DAB development under microscope to prevent overstaining

> - DAB is a potential carcinogen - handle with care and dispose properly

Required Materials

Reagents

- Xylene (for paraffin sections)
- 100%, 95%, 70%, 50%, 20% Ethanol
- ddH_2O (distilled water)
- Citrate antigen retrieval buffer

  Solution A: 0.1M citric acid
  Solution B: 0.1M sodium citrate
  Mix: 9 mL A + 41 mL B
- Filtered PBS
- PBS-T (PBS + 0.1% Triton X-100)
- 30% H_2O_2 (hydrogen peroxide)
- 10% serum (matched to secondary antibody host)
- Primary antibody
- Secondary antibody (biotinylated)
- ABC kit (Avidin-Biotin Complex)
- DAB kit (3,3'-Diaminobenzidine)
- Hematoxylin (for counterstaining)
- 2% Acetic acid
- Bluing solution
- Histomount mounting medium

Equipment

- Coplin jars
- Microwave
- Humidifying chamber
- PAP pen (hydrophobic barrier pen)
- Coverslips

- Timer
- 4°C refrigerator or cold room
- Light microscope (for monitoring DAB)
- Pipettes and tips
- Kimtech wipes

Solution Recipes

Citrate Buffer (pH 6.0):

Solution A: 19.21 g citric acid anhydrous in 1L H_2O (or 21.01 g citric acid monohydrate)

Solution B: 29.41 g sodium citrate dihydrate in 1L H_2O

Working solution: 9 mL A + 41 mL B

0.6% H_2O_2 in PBS:

1 mL 30% H_2O_2 + 49 mL filtered PBS

PBS-T:

39 mL filtered PBS + 1 mL 10% Triton-X

10% Serum Block:

100 µL serum + 900 µL PBS-T

2% Serum (Antibody Dilution):

20 µL serum + 980 µL PBS-T

DAB Solution:

2.5 mL H_2O + 5 µL 30% H_2O_2 + 1 drop buffer + 1 drop DAB reagent

⚠️ Prepare immediately before use!

Select Protocol Day:

Day 1 (4 hours + overnight) | Day 2 (4 hours)

Day 1: Sample Preparation and Primary Antibody

Total time: ~4 hours + overnight incubation

Select Sample Type:

Paraffin Sections | Cryo Sections

1 Deparaffinization (~30 minutes)

- [ ] Xylene - 10 minutes
- [ ] Xylene - 3 minutes
- [ ] Xylene - 3 minutes
- [ ] 100% EtOH - 2 minutes
- [ ] 100% EtOH - 2 minutes
- [ ] 95% EtOH - 1 minute
- [ ] 70% EtOH - 1 minute
- [ ] 50% EtOH - 1 minute
- [ ] 20% EtOH - 1 minute
- [ ] Transfer to ddH_2O

2 Antigen Retrieval (~1 hour)

- [ ] Place slides in coplin jar with citrate buffer, sit 10 min at RT
  ⚠️ Have a second jar with buffer only to microwave alongside slides
- [ ] Microwave 2.5 min at 50% power (power level 5)

- [ ] Microwave 2.5 min at power level 4
- [ ] Microwave 2.5 min at power level 4
- [ ] Microwave 2.5 min at power level 4
- [ ] Remove lid and allow to cool AT LEAST 30 minutes
  ⚠️ Critical: Do not rush cooling step

1 Antigen Retrieval (~15 minutes)

- [ ] Microwave citrate buffer 2.5 min at power level 5
- [ ] Pour heated buffer over slides
- [ ] Microwave slides 2.5 min at power level 4
- [ ] Allow to cool for 5 minutes

3 Peroxide Quench (~1 hour)

- [ ] Wash with filtered PBS 5 minutes
  Pour citrate out of coplin jar, pour PBS in
- [ ] Prepare 0.6% H_2O_2 in filtered PBS (1 mL 30% H_2O_2 + 49 mL PBS)
  💡 H_2O_2 is stored in 4°C fridge
- [ ] Treat slides with 0.6% H_2O_2 for 30 minutes
  Pour PBS out, pour H_2O_2/PBS in

- [ ] **Wash 10 min with PBS-T 2× (in humidifying chamber)**
  PBS-T: 39 mL filtered PBS + 1 mL 10% Triton-X

4 Blocking (1 hour)

- [ ] **Prepare 10% serum in PBS-T (100 µL serum + 900 µL PBS-T)**
  💡 Use serum from same species as secondary antibody host (usually goat)
- [ ] **Cover slides (approx. 300 µL per slide) with 10% serum for 1 hour**

5 Primary Antibody (~30 min + Overnight)

- [ ] **Wash slides with PBS-T 5 min 3×**
- [ ] **Dilute primary antibody in 2% serum (20 µL serum + 980 µL PBS-T)**
  💡 Dilution varies by antibody - see Antibody Info tab
- [ ] **Apply primary antibody to slides in humidifying chamber**
- [ ] **Incubate overnight at 4°C**
  ⚠️ Ensure humidifying chamber is well-sealed

Day 2: Detection, Development, and Mounting

Total time: ~4 hours

1 Secondary Antibody (~1 hr 20 min)

- [ ] **Wash slides with PBS-T 5 min 3×**

- [ ] **Dilute secondary antibody in 2% serum**
  💡 Dilution varies by antibody - see Antibody Info tab

- [ ] **Overlay slides and incubate at room temp for 1 hour**

2 ABC Complex (~1 hr 20 min)

- [ ] **Prepare AB Solution AT LEAST 30 min before use**
  5 mL PBS-T + 1 drop A + 1 drop B, invert to mix, store in drawer

- [ ] **Wash slides with PBS-T 5 min 3×**

- [ ] **Overlay slides with AB solution, incubate at room temp 1 hour**

3 Color Development (DAB)

> ⚠️ **CRITICAL STEP - DAB Safety**
> DAB is a potential carcinogen. Wear gloves, work in fume hood, and dispose properly as hazardous waste.

- [ ] **Wash slides with PBS-T 5 min 3×**

- [ ] **Prepare DAB IMMEDIATELY before use**
  2.5 mL H_2O + 5 µL 30% H_2O_2 + 1 drop buffer + 1 drop DAB reagent

- [ ] **Overlay slides with DAB, monitor under microscope**
  ⚠️ Time and observe color development - typically 1-5 minutes

- [ ] **Place slides in H_2O to stop reaction**

4 Counterstaining (~15 min)

- [ ] Place slides in hematoxylin for NO LONGER than 10 seconds
  ⚠️ Over-staining will obscure DAB signal
- [ ] Wash slides with running H_2O 5 minutes
- [ ] Dip slides in 2% acetic acid 10×
- [ ] Dip slides in ddH_2O 10×
- [ ] Place slides in bluing solution 1 minute
- [ ] Wash slides with running H_2O 3 minutes

5 Dehydration (~30 min)

- [ ] 20% EtOH - 1 minute
- [ ] 50% EtOH - 1 minute
- [ ] 70% EtOH - 1 minute
- [ ] 95% EtOH - 1 minute
- [ ] 100% EtOH - 1 minute
- [ ] 100% EtOH - 2 minutes
- [ ] 100% EtOH - 2 minutes
- [ ] Xylene - 5 minutes

- [ ] Xylene - 5 minutes
- [ ] Xylene - 5 minutes

6 Mounting and Coverslip

- [ ] Wipe xylene off slides with Kimtech wipes
  ⚠️ DO NOT wipe tissue sample
- [ ] Add a couple drops of Histomount
- [ ] Place coverslip gently
- [ ] Use clean pipette tip to push out air bubbles
- [ ] Allow slides to dry overnight at room temperature

Antibody Dilutions and Details

Below are specific antibody protocols used in our lab. Always optimize dilutions for your specific application.

ATIC (5-Aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase)

Storage & Location:

- Stored in -20°C freezer
- Antibody box A-D

Serum:

- Use goat serum for blocking

- Goat serum stored in -20°C freezer near TC

Dilutions:

- **Primary antibody:** 1:100 (10 µL per 1 mL solution)
- **Secondary antibody:** Goat anti-mouse, 1:200 (5 µL per 1 mL solution)
- Secondary stored in 4°C fridge, antibody box

CC3 (Cleaved Caspase-3)

Storage & Location:

- Stored in -20°C freezer
- Antibody box A-D

Application:

- Apoptosis marker
- Commonly used for cell death studies

Dilutions:

Dilution to be optimized (protocol cut off in original document)

> 💡 **General Guidelines for New Antibodies:**
>
> - Always start with manufacturer's recommended dilution
> - Run a dilution series (e.g., 1:50, 1:100, 1:200, 1:500) to optimize
> - Include positive and negative control tissues
> - Secondary antibody should be biotinylated for ABC method
> - Match secondary antibody host to serum used for blocking
> - Store antibody aliquots at -20°C to avoid freeze-thaw cycles

Troubleshooting Guide

High Background Staining

Possible Causes:

- Insufficient blocking
- Antibody concentration too high
- Inadequate washing
- Endogenous peroxidase not quenched
- DAB development too long

Solutions:

- Increase blocking time or serum concentration (try 20%)
- Increase antibody dilution (lower concentration)
- Extend wash times and increase number of washes
- Increase H_2O_2 quenching time
- Monitor DAB more carefully under microscope

No Signal or Weak Signal

Possible Causes:

- Antibody concentration too low
- Primary or secondary antibody not working
- Antigen not present or masked
- Insufficient DAB development
- ABC solution not prepared correctly

Solutions:

- Decrease antibody dilution (higher concentration)
- Test antibodies with positive control tissue
- Optimize antigen retrieval (try different methods)
- Extend DAB development time
- Ensure ABC solution was prepared 30+ min before use

Excessive Counterstain

Possible Causes:

- Hematoxylin incubation too long

- Hematoxylin solution too concentrated

Solutions:

- Reduce hematoxylin time to 5-10 seconds
- Use fresh hematoxylin or dilute if too strong
- Can differentiate in acid alcohol if caught early

DAB Precipitate on Slides

Possible Causes:

- DAB solution not mixed well
- DAB solution prepared too far in advance
- Dust or debris on slides

Solutions:

- Mix DAB solution thoroughly
- Always prepare DAB immediately before use
- Filter DAB solution if necessary
- Ensure slides are clean and dust-free

Tissue Falls Off Slides

Possible Causes:

- Slides not positively charged
- Microwave power too high
- Excessive agitation during washes

Solutions:

- Use positively charged slides only
- Reduce microwave power during antigen retrieval
- Be gentle during washing steps
- Bake slides at 60°C for 30-60 min before staining

Safety Information

- **DAB is a potential carcinogen** - always wear gloves, work in fume hood, dispose as hazardous waste
- Always work with xylene in a fume hood - fumes are toxic
- Wear appropriate PPE (lab coat, gloves, safety glasses)
- Handle antibodies with care - some may contain sodium azide preservative
- H_2O_2 can cause burns - handle with care
- Be cautious with hot liquids during antigen retrieval
- Follow proper storage conditions for antibodies (typically 4°C or -20°C)

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About This Protocol

Immunohistochemistry (IHC) staining protocol using ABC method with DAB chromogen for detecting specific proteins in tissue sections.

Quick Links

Protocol Overview

Antibody Info

Troubleshooting

Contact

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