Protocol

Detecting Horseradish Peroxidase-Labeled Cells

Scott J. Rodig

A range of substrates is available for detection of cells labeled with horseradish peroxidase (HRP). Diaminobenzidine (DAB) is the most commonly used substrate and one of the most sensitive. It yields an intense brown product that is insoluble in both water and alcohol. DAB staining is compatible with a wide range of common histological stains. DAB can be made more sensitive by adding metal salts such as cobalt or nickel to the substrate solution. The reaction product is slate gray to black, and the products are stable in both water and alcohol. DAB/metal staining is compatible with a wide range of common histological stains. Alternatively, chloronaphthol gives a blue-black product and aminoethylcarbazole (AEC) yields a red product. These substrates are less sensitive than DAB and the products are soluble in alcohol, but they can be used if the DAB reaction gives too high a background or if alternative product colors are required.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Cells labeled with horseradish peroxidase (HRP)-labeled antibodies

H₂O₂ (3%)

H₂O₂ is supplied as a 30% solution and should be stored at 4°C. It will last ~1 mo.

Sodium acetate (0.1 M, pH 5.2) (for detection using AEC)

Substrate for detection of HRP

Aminoethylcarbazole (AEC; 0.4% in DMF) (see Steps 20–26)

*Prepare a stock solution of AEC by dissolving 400 mg of aminoethylcarbazole in 100 mL of *n,n*-dimethyl formamide (DMF).*

Chloronaphthol (see Steps 14–19)

Prepare a stock solution of chloronaphthol by dissolving 0.3 g of chloronaphthol in 10 mL of absolute ethanol and storing at –20°C.

Diaminobenzidine (DAB) tetrahydrochloride (see Steps 1–6 and 7–13)

Nickel chloride or cobalt chloride (0.3%, w/v) in H₂O (see Steps 7–13)

Tris buffer (0.05 M, pH 7.6) (for detection using DAB or chloronaphthol)

Equipment

Whatman No. 1 filter paper

From the Antibodies collection, edited by Edward A. Greenfield.

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METHOD

Detecting HRP-Labeled Cells Using DAB

Figure 1 shows the results of DAB-based chromogenic staining of formalin-fixed, paraffin-embedded human tonsil.

1. Dissolve 6 mg of DAB in 10 mL of 0.05 M Tris buffer (pH 7.6).
2. Add 0.1 mL of 3% H₂O₂.
3. If a precipitate appears, filter through Whatman No. 1 filter paper (or equivalent).
4. Apply to specimen and incubate for 1–20 min. Stop the reaction by washing in water.
5. (Optional) Counterstain if necessary (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).
6. Mount in DPX (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).

Detecting HRP-Labeled Cells Using DAB and Metal Salts

7. Dissolve 6 mg of DAB in 9 mL of 0.05 M Tris buffer (pH 7.6).
8. Add 1 mL of a 0.3% (w/v) stock solution of nickel chloride in H₂O (the same amount of cobalt chloride can be used as an alternative).
9. Add 0.1 mL of a 3% solution of H₂O₂ in H₂O.
10. If a precipitate appears, filter through Whatman No. 1 filter paper (or equivalent).
11. Apply to specimen and incubate for 1–20 min at room temperature. Stop the reaction by washing in H₂O.

FIGURE 1. Human tonsil stains. Human tonsil stained with hematoxylin and eosin (A), anti-CD20 (B), anti-CD3 (C), and anti-Ki67 (D). The images show reactive germinal centers that are rich in B cells (CD20⁺) compared with T cells (CD3⁺) and are sites of cellular proliferation (Ki67⁺). Brown coloration in B–D indicates positive staining with antibody by IHC. Hematoxylin (but no eosin) was used as a counterstain in B–D. Photographed at 100× final magnification.

12. (Optional) Counterstain if necessary (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).
13. Mount in DPX (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).

Detecting HRP-Labeled Cells Using Chloronaphthol

14. Add 100 μ L of chloronaphthol stock with stirring to 10 mL of 0.05 M Tris (pH 7.6).
15. Add 0.1 mL of 3% H_2O_2 in H_2O .
16. Remove the white precipitate that forms by filtering through Whatman No. 1 filter paper (or equivalent).
17. Apply to specimen and incubate for 10–40 min at room temperature. Stop the reaction by washing in H_2O .
18. (Optional) Counterstain if necessary. Only aqueous stains can be used (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).
19. Mount in Gelvatol or Mowiol (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).

Detecting HRP-Labeled Cells Using AEC

20. Add 1.0 mL of the 0.4% AEC solution to 15 mL of 0.1 M sodium acetate buffer (pH 5.2) with stirring.
21. Add 0.15 mL of 3% H_2O_2 in H_2O .
22. Filter through a Whatman No. 1 filter.
23. Apply the reagent to the cell specimen and incubate for 10–40 min at room temperature.
24. Stop the reaction by washing in H_2O .
25. (Optional) Counterstain if necessary. Only aqueous stains can be used (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).
26. Mount in Gelvatol or Mowiol (see Protocol: **Counterstaining, Mounting, and Photographing Stained Cells** [Rodig 2019]).

The AEC product fades if oxidized. Take special care to avoid air bubbles upon mounting.

DISCUSSION

Tyramide signal amplification (TSA) is a proprietary technique used to increase the sensitivity of detecting low numbers of antibody–antigen complexes in tissues or cells (Zaidi et al. 2000; Krieg and Halhuber 2010). The basis for activity is a time-dependent, HRP-catalyzed covalent attachment of labeled (biotin/fluorescent dye conjugated) molecules of tyramide to tyrosine residues on the nearby HRP-conjugated antibody or protein. The local deposition of the conjugate (biotin/fluorescent dye) is then detected by standard means (such as streptavidin–HRP/DAB or direct visualization of the fluorescent dye). Although such catalyzed amplification techniques can increase the sensitivity of detecting antibody–antigen complexes, they often come at the expense of increased background. A careful set of controls is necessary.

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