Video Article

Immunohistochemistry: Paraffin Sections Using the Vectastain ABC Kit from Vector Labs

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Abstract

Immunohistochemistry (IHC) is a valuable technique utilized to localize/visualize protein expression in a mounted tissue section using specific antibodies. There are two methods: the direct and indirect method. In this experiment, we will only describe the use of indirect IHC staining. Indirect IHC staining utilizes highly specific primary and biotin-conjugated secondary antibodies. Primary antibodies are utilized to discretely identify proteins of interest by binding to a specific epitope, while secondary antibodies subtract for non-specific background staining and amplify signal by forming complexes to the primary antibody. Slides can either be generated from frozen sections, or paraffin embedded sections mounted on glass slides. In this protocol, we discuss the preparation of paraffin-embedded sections by dewaxing, hydration using an alcohol gradient, heat induced antigen retrieval, and blocking of endogenous peroxidase activity and non-specific binding sites. Some sections are then stained with antibodies specific for T cell marker CD8 and while others are stained for tyrosine hydroxylase. The slides are subsequently treated with appropriate secondary antibodies conjugated to biotin, then developed utilizing avidin-conjugated horseradish peroxidase (HRP) with Diaminiobenzidine (DAB) as substrate. Following development, the slides are counterstained for contrast, and mounted under coverslips with permount. After adequate drying, these slides are then ready for imaging.

Protocol

Staining of paraffin sections

1. Dewax slides with Xylene (Fisher $X3^S$-4) 3x for 5 minutes each (change xylene every month depending on use, xylene is TOXIC). Use dishes and covers for 20 slides from Fisher ref 08-812-1A and racks made to fit these dishes. 200 ml liquid per dish.
2. Hydrate through alcohol gradient as follows (change gradient every 2 weeks):
   - 2x in 100% ethanol (Fisher # A406-20) for 2 min each
   - 2x in 95% ethanol for 2 min each
   - 1x in 70% ethanol for 2 min
   - 1x in 50% ethanol for 2 min
   - 1x in 30% ethanol for 2 min
   - 1x ddH_2O for 2 min
3. Soak in DPBS for 5 min (DPBS = Dulbecco's modified Phosphate Buffered Saline with Ca^{2+} and Mg^{2+}).

Antigen recovery:

1. Pre-heat the Na-Citrate buffer (10 mM, pH 6.5) in the microwave.
2. Cook the slides for 15 min in the microwave. The slides should never dry so check every min and add Na-Citrate when necessary.
3. Let cool at room temperature.
4. Block endogenous peroxidase activity with 1% H_2O_2 in DPBS for 20 min (1.5 ml of 30% stock Sigma H-1009 in 50 ml DPBS).
5. Soak in DPBS 3 x 5 min.
6. Block non-specific sites by incubating overnight at +4°C in DPBS + 5% bovine serum albumin (BSA) + 0.1% Na Azide + 5% serum.
   Note: Choice of serum will depend on species in which antibodies used for staining were made. Block with serum from the species in which the secondary Ab (conjugated to biotin) was made. If this is not available, use serum from a species different from the one in which the primary Ab was made.
7. Block the biotin sites with the avidin/biotin blocking kit (Vector laboratories catalog # SP-2001):
   - Incubate with Avidin D for 15 min. Rinse briefly with DPBS.
   - Incubate with the biotin solution for 15 min.
8. Incubate in primary Ab for 2 hrs at room temperature, in DPBS + 2% BSA + 0.1 % NaAzide + 2% serum (same as for Blocking step).
9. Soak in PBS 3 x 5 min.
10. Incubate with the secondary Ab conjugated with biotin for 1 hour at room temperature in DPBS + 2% BSA + 0.1% Na Azide + 2% serum (same as used for the blocking step).
11. Prepare ABC reagent at the same time because it has to develop for at least 30 min before use!!! (see Materials)
12. Prepare in a 50 ml conical tube. In 15 ml DPBS (no serum, no azide) add 3 drops of reagent A, mix and add 3 drops of reagent B and mix. Avoid squeezing the bottles, rather wait for the drops to form on their own. ABC kit Vectastain PK-6100 from Vector Labs.
13. Soak in DPBS 3 x 5 min.
14. Incubate with ABC reagent for 30-45 min at room temperature.
15. Soak in DPBS 3 x 5 min.

16. Prepare DAB peroxidase substrate (Vector Labs # SK-4100) in 5 ml ddH_2O in a glass vial **immediately before use** (see Materials)
    - 2 drops of buffer stock solution, mix
    - 4 drops of DAB, mix (should become slightly brown)
    - 2 drops H_2O_2, mix
17. Drop the DAB substrate on top of the slides and watch for brown staining.
18. Dip slides into ice + tap water to stop the reaction.
19. Rinse under cold tap water for 5 min.
20. Counterstain with Hematoxylin (20 sec; Fisher CS401-D) and rinse in tap water until water comes out clear.
21. Dehydrate through alcohol gradient starting at 30% ethanol up to 100% ethanol (2 min each).
22. Soak in xylene 3 x 5 min.
23. Mount with Permount (Fisher # SP15-100): put some above the section on the slide and press slowly onto the section with a coverslip without air bubbles. Do not move the coverslip until completely dry, which takes ~ 24 hours.

References

