

Protocol: Double Immunofluorescent Labeling Using Two Primary Antibodies From Different Species

Staining for First Antigen

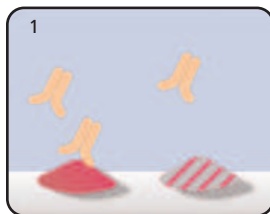
1. **Preparation of tissue.** Fix sections with the appropriate fixative for the antigen under study (Please see Note 1).
2. Air dry sections.
3. Wash sections 2 x 2 minutes in buffer (PBS).
4. **Avidin/biotin blocking step.** Perform Avidin/Biotin blocking if required (Avidin/Biotin Blocking Kit, Cat. No. SP-2001). Incubate sections with Avidin Solution for 15 minutes. Rinse briefly with buffer, then incubate in the Biotin Solution for 15 minutes. Wash sections 2 x 2 minutes in buffer. This blocking step may be eliminated if suitable controls have determined this step to be unnecessary.
5. **Protein blocking step.** Incubate sections for 20 minutes with buffer containing 5% normal blocking serum (NS) which was prepared from the species in which the secondary antibody is made.
6. Blot excess serum from sections.
7. **Primary antibody.** Incubate sections with the first primary antibody diluted in appropriate antibody diluent (buffer containing 5% NS) using an appropriate concentration and length of incubation.
8. Wash for 5 minutes in buffer.
9. **Secondary antibody.** Incubate sections for 30 minutes with biotinylated secondary antibody (5-10 $\mu\text{g/ml}$ diluted in buffer containing 5% NS).
10. Wash slides for 5 minutes in buffer.
11. **Avidin conjugate.** Incubate sections with Fluorescein Avidin DCS (15-20 $\mu\text{g/ml}$ diluted in buffer) for 5-10 minutes.
12. Wash slides for 5 minutes in buffer.

Staining for Second Antigen

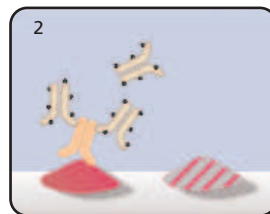
13. **Avidin/biotin blocking step.** Apply an Avidin/Biotin block according to Step 4. (This step must be done to prevent the interaction of the second set of labeling reagents with the first set of labeling reagents).
14. **Protein blocking step.** Incubate sections for 20 minutes with 5% NS.
15. Blot excess serum from sections.
16. **Primary antibody.** Incubate sections with second primary antibody diluted in appropriate antibody diluent (buffer containing 5% NS) using an appropriate concentration and length of incubation.
17. Wash slides for 5 minutes in buffer.
18. **Secondary antibody.** Incubate sections for 30 minutes with biotinylated secondary antibody (5-10 $\mu\text{g/ml}$ diluted in buffer containing 5% NS).
19. Wash slides for 5 minutes in buffer.
20. **Avidin conjugate.** Incubate sections with Texas Red[®] Avidin DCS (15-20 $\mu\text{g/ml}$ diluted in buffer) for 5-10 minutes.
21. Wash slides for 5 minutes in buffer.
22. Mount with the appropriate VECTASHIELD[®] mounting media.
23. Observe under a fluorescence microscope.

NOTES:

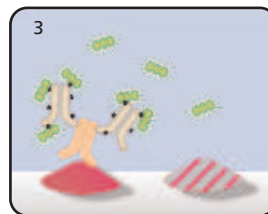
1. Aldehyde-fixed tissues (e.g. formalin) tend to be autofluorescent and may make interpretation of specific fluorescein signal difficult.



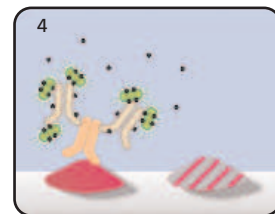
1 Add first primary antibody.



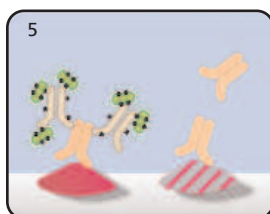
2 Add biotinylated secondary antibody.



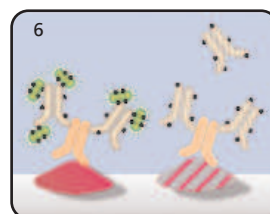
3 Add Fluorescein Avidin DCS.



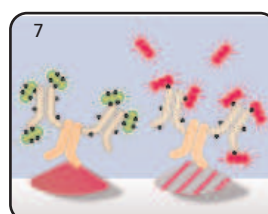
4 Add avidin/biotin block.



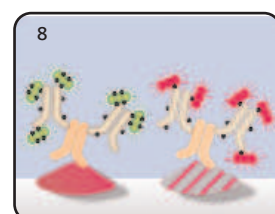
5 Add second primary antibody.



6 Add biotinylated secondary antibody.



7 Add Texas Red[®] Avidin DCS.



8 Mount and observe.