STEPWISE IMMUNOPEROXIDASE PROTOCOL: (Steps 12 through 17 are carried out in DAKO Autostainer Plus)

1. Cut paraffin sections to 3-4 microns.
2. Melt paraffin by placing slides in either a 58ºC oven for 5 minutes or preferably in a 37ºC oven overnight.
3. Dewax slides in xylene, 1 bath x 10 minutes.
4. Rehydrate slides in decreasing ethanol solutions, 1 minute each, 2 baths of absolute, 2 baths of 95%, 1 bath of 90%.
5. Block endogenous peroxidase with a solution of 6% Hydrogen Peroxide (H₂O₂) for 3 minutes.
6. Rinse slides in tap water for 1 minute.
7. Place rack in TBS (DAKO S1968) bath for 1 minute.
8. Prepare target retrieval (TR): In a staining dish: 20 ml target retrieval (DAKO S1699) plus 180 ml DH₂O. Add DH₂O to pressure cooker and turn it on. Place staining dish containing target retrieval solution inside the cooker and let it heat for 20 minutes. TR solution should heat to 90ºC.
9. Take out staining dish from the pressure cooker and place slides inside dish (use gloves) and heat for 20 minutes.
10. Cool slides down in same container for 20 minutes.
11. Place slides in PBS buffer at room temperature (in this step you can stop the technique and keep them in the buffer, from 2 minutes to 18 hours and then continue with the technique).
12. Biotin blocking system (DAKO X0590): The tissue sections are incubated with (1) Avidin Solution for 6 minutes. The avidin solution is then rinsed off and the slides are incubated with (2) Biotin Solution for 6 minutes. The biotin solution should be washed off before application of the first step of the staining procedure.
13. Add specific primary antibody to each slide, incubate for 30 minutes in a humidity chamber.
14. Place slides back in rack and submerge rack in TBS bath 2 minutes. Dry excess TBS off each slide, and add the linking solution (DAKO KO690 LSAB + Kit, biotinylated anti-mouse, anti-rabbit and anti-goat). Incubate for 22 minutes in humidity chamber.
15. Place slides back in rack and submerge in TBS bath 2 minutes; then, DH₂O 3 minutes and then TBS 2 minutes.
16. Dry excess TBS off slide. Add streptavidin-peroxidase conjugate and incubate for 22 minutes in humidity chamber.
17. Submerge rack in TBS bath for 2 minutes. Prepare DAB (DAKO K3468) chromogen for next step (1 drop concentrated DAB per 1 ml substrate). Place rack in DAB solution for 10 minutes. Place slides into fresh TBS. Rinse slides for 4 minutes.
18. Dry slides and counterstain with Harris hematoxylin (from 15 to 40 seconds).

**NOTE:** For nuclear antigens: Dry excess TBS from slides and apply 1% Cupric Sulfate for 1 minute. Rinse slides in tap water for 2 minutes. Place slides in 0.2% fast green for 1 or 2 seconds.

19. Dehydrate through gradient alcohols, clean in xylene and coverslip.