PROTOCOL FOR PARAFFIN SECTIONS FOR IMMUNOSTAINING

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Solution Preparation

1. Endogenous enzyme blocking: 3% H_2O_2 in methanol.
2. Protein blocking: 5% horse serum + 1% goat serum in PBS.
3. 0.2% Stock Light Green (Fisher, 03382-255): Light Green, SF yellowish (0.2g)/ ddH_2O (100cc)/ Glacial Acetic Acid (0.2cc).
4. Light Green Working solution: Light Green Stock 10 cc + ddH_2O 50cc.
5. 0.1M Citrate Buffer: 18 ml soln A+ 82 ml soln B+ 900ml water, Soln A = 0.1 Mcitric Acid: 9.72 g citric acid in 500 ml H_2O, Soln B = 0.1 M Sodium Citrate: 14.705g sodium citrate in 500ml H_2O.
6. Acetic Acid : Chloroform (1:1).
7. 2% Acetic Acid.
8. 1.5% NH_4OH in 70% ETOH.

Deparafiinization

1. Dunk the section in Xylene, 5 minutes $\times$ 3 (=each time for 5 min, total 3 times, similar as below).
2. Dunk in 100%ETOH, 5 minutes $\times$ 2.
3. Dunk in 95%ETOH, 5 minutes $\times$ 2.
4. Dunk in ddH_2O for 1 minute.
5. Boil the sections in citrate buffer @ 95^0C for 10 minutes, cool down @ RT (about 30 minutes).

Fixation

1. Dunk the sections in cold acetone for 5 minutes.
2. Dunk the sections in acetone + chloroform (1:1) for 5 minutes.
3. Dunk the sections in Acetone for 5 minutes.
4. Washing with PBS, 2 minutes $\times$ 2.
5. Place slides in a humidity chamber with the sections covered with PBS.

Blocking endogenous peroxidase

1. Incubate the slides in endogenous enzyme blocking solution for 12 minutes.
2. Wash with PBS, 2 minutes x 3.
3. Encircle specimen with Pap-pen.

Avidin/Biotin Blocking

1. Add 4 drops of avidin blocking solution (from Blocking Kit, SP-4100, Vector Laboratories, Inc, Burlingame, CA 94010) into 1 ml protein blocking solution.
2. Apply the Avidin blocking solution to specimen for 20 minutes.

3. Wash with PBS, 2 minutes × 3.
4. Add 4 drops of Biotin blocking solution (from the Blockingg Kit mentioned above) in 1 ml protein blocking solution, used for diluting the primary antibody.

Primary and secondary antibodies

1. Add the primary antibody diluted in protein blocking solution, incubate the slides for 1-3 hr. at RT or overnight at 4^0C
2. Washing with PBS, 2 minutes × 3.
3. Incubate the slides in protein blocking solution for 10 minutes.
4. Add the secondary antibody diluted in protein blocking solution.
5. Incubate the slides for 1-3 hr. at RT
6. Washing with PBS, 2 minutes × 3.

Detection

1. Apply 2 drops of "Linker" (MULTILINK: super sensitive Biotinylated IgG, HK-340-5K, ***Biogenex***, San Ramon,CA94583).
2. Washing with PBS, 2 minutes × 3.
3. Add 2 drops of "Label" (LABEL: peroxidase conjugated streptavindin, ***Biogenex***).
4. Washing with PBS, 2 minutes × 3.
5. Develop the reaction with DAB: take 1ml of "DAB/Metal concentrate" into 9 ml of "Stable Peroxide Substrate Buffer", then apply to the color development. (PIERCE, No.1 856090).
6. Counterstain with Light Green working solution for 30-60 minutes.
7. Dehydrate through alcohols: 10 tips in 95% ETOH, and 10 tips in 100% ETOH, then 20 tips in Xylene × 2.
8. Mount the slide with permount and cover slips.

For H.E staining, All of the steps are similar but:
In the step of counterstaining, dunk the slides in hematoxylin 5 min @RT, washing 1 min with water, rinsing the slides for 10 tips in 2% acetic acid, and blocking for 1 min in 1.5%NH_4OH in 70%ETOH

Materials needed

1. Plastic sinkers for solutions
2. Glass sinkers for sinking the slide
3. Slide holder
4. 95^{o}C water-bath
5. chamber
6. slides
7. cover slips
8. slide box
9. Pap-pen

Agents needed

1. Mathanol
2. H_2O_2
3. Horse and goat serum
4. PBS
5. Lgiht Green
6. SF yellowish
7. Acetone
8. Ethanol
9. Xylene
10. Permount
11. Hematoxylin