Immunohistochemical staining for Paraffin Sections

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A. Solutions

1) Endogenous enzyme blocking solution: 3% Hydrogen Peroxide (dilute from 30% stock) in methanol
2) 30% Hydrogen Peroxide (Sigma 7722-81-1)
3) **Protein Blocking** solution: May use undiluted Goat, Horse, or Calf serum (**keep in mind primary Antibodies when choosing; e.g., you can't use goat serum for blocking if your primary antibody was made from goat**).
4) 0.2% Light Green (Fisher 08382-255): 0.2 g Light Green, SF yellowish in 100 ml dH_20 and 0.2 ml Glacial Acetic Acid
5) Light Green Working Solution: 1 ml Light Green Stock and 50 cc dH_20
6) 0.1 M citrate buffer: 18 ml Solution A and 82 ml Soln B in 900 ml dH_20
   Solution A (0.1 M Citric Acid): 9.72 g Citric Acid in 500 ml dH_20
   Solution B (0.1 M Sodium Citrate): 14.705 g Sodium Citrate in 500 ml dH_20
7) Chloroform
8) Acetone
9) Xylene

B. Deparaffinization

1) Dunk section in Xylene, 5 min x 3
2) Dunk in 100% Ethanol, 5 min x 2
3) Dunk in 95% Ethanol, 5 min x 2
4) Dunk in dH_20 for 1 min
5) Microwave citrate buffer and bring solution and slides to a boil (usually 2 minutes). Then microwave with the slides for 10 min at 20% power. Goal is to keep samples around 95 ^{o} C.
6) Let cool down at RT for approx 30 min for renaturation of proteins (or so-called **antigen retrieval**).

C. Fixation

1) Dunk in cold Acetone (4 ^{o} C) for 5 min
2) Dunk in acetone:chloroform (1:1) for 5 min at RT
3) Dunk in acetone for 5 min at RT
4) Wash in PBS, 2 min x 2

D. Endogenous Peroxidase Blocking

1) Incubate in **endogenous** blocking solution for 12 min
2) Wash with PBS, 2 min x 3
3) Encircle specimen with Pap-Pen or Immuno-pen

E. Protein Blocking

1) Block specimen with a few drops of **Protein Blocking solution** for 30-60 min. May block longer for reducing background.
2) Pour off solution.

F. Primary Antibody

1) Add primary antibody diluted in **Protein Blocking solution** or 4% milk. Add to slides and incubate for 1-3 hrs at RT or O.N. at 4 ^{o} C
2) Wash with PBS 2 min x 3

G. Biogenex Multilink (or Link) Detection System

1) *Can block with **Protein Blocking solution** for 15 min. before adding secondary Ab to reduce background*. **However, for primary antibodies with a weaker reactivity, this step could reduce the detection sensitivity**. For S100A6, block before adding secondary Ab.
2) Dilute a specific **Link** (e.g., for goat IgG) or **Mulitlink** (for mouse, rat, and rabbit IgGs) solution (Biogenex) 1:2 in **Protein Blocking solution** (or PBS if blocking done in step one) and add to slide.
3) Incubate at RT for 20 min
4) Remove Ab and wash in PBS for 4 min x 4
5) Add a few drops of **Label Solution** (diluted 1:2 in PBS) and incubate at RT for 20 min
6) Remove Ab and wash in PBS for 4 min x 4

H. DAB Developing

1) Prepare developing solution (Vector System) (can prepare ½ the amount below to save reagents).

   5 ml dH_20
   2 drops of Buffer solution

4 drops of DAB Rxn solution
2 drops of H_2O_2
2 drops of NiCl solution

2) Add a few drops to the slides
3) Watch slides develop under light microscope with attention to the negative and positive controls. **Stop the reaction when there is adequate staining and minimal background**. This was approx. 20 min for S100A6 and A2. Need to gently shake slides every 5-10 minutes to prevent precipitates from collecting on tissue.
4) Stop staining by washing slides in PBS, 2 min x 2

I. Counter Staining

1) Prepare Light Green working solution
2) Allow counter-staining for 1-3 min (or slightly longer), but you have to watch negative controls very carefully (***Note: darker Light Green staining will obscure the IHC staining results, so it's better to stop countstaining and you can always re-add Light Green for longer staining***).
3) Dehydrate through alcohol:
   10 dips in 95% Ethanol
   10 dips in 100% Ethanol
   20 dips in Xylene x2

J. Mount Slides

1) Mount slides with Permount (Fischer SP15-500) and cover slips
2) Allow air-drying for 24-48 hrs.