

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 dH₂O and 0.2 ml Glacial Acetic Acid
- 5) Light Green Working Solution: 1 ml Light Green Stock and 50 cc dH₂O
- 6) 0.1 M citrate buffer: 9 ml Solution A and 41 ml Soln B in 450 ml dH₂O
Solution A (0.1 M Citric Acid): 9.72 g Citric Acid in 500 ml dH₂O
Solution B (0.1 M Sodium Citrate): 14.705 g Sodium Citrate in 500 ml dH₂O
- 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 dH₂O for 1 min
- 5) Microwave 500 ml citrate buffer in a covered container and bring to a boil. Place slide holder (with slides) into the buffer and close the lid. Goal is to keep samples around 95 ° C.
- 6) Remove container from the microwave and let cool down at RT on the bench top for approx 30 min for renaturation of proteins (**antigen retrieval**).

C. Fixation

- 1) Dunk in cold Acetone (4 ° 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 at RT for 12 min (**Note in Methanol**)
- 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) Aspirate 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 ° 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. Aspirate off solution.
- 2) Dilute a specific **Link** (e.g., for goat IgG) or **Multilink** (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 fresh developing solution (Vector System) (can prepare ½ the amount below to save reagents).

2.5 ml ddH₂O

1 drops of Buffer solution

2 drops of DAB Rxn solution

1 drops of H₂O₂

1 drops of NiCl solution (For desired black/gray staining)

- 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. 10 min for CTGF. May need to gently shake slides every 5-10 minutes to prevent precipitates from collecting on tissue.
- 4) Stop staining by placing slides in dH₂O

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 counterstaining 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.