

Protocol for immunohistochemical staining of Paraffin sections to unmask epitopes (AR) with amplification, using catalyzed signal amplification

A. Tissue Controls:

1. Positive control: Cells or tissue expected to be positive. Positive controls may also be the cells that are known to express the epitope, that are grown in tissue culture up to about 100 million, taken and made into a pellet, fixed, paraffin embedded and sectioned.
2. Negative control: Cells or tissue expected to be negative.
3. Blocking peptide (see below)

B. Reagent controls:

1. 1% BSA/PBS reagent negative control for secondary reagents
2. Rabbit IgG Dako Cat.# 1505 Negative control reagent at 5 ug/ml
3. Rabbit anti human vWf Dako Cat# 0936 (pre-diluted). Positive for blood vessels in tissue.
4. Mouse anti Vimentin Dako Catalog# N1521 prediluted, ready to use, as a technical positive control when using mouse antibodies as test
5. Mouse IgG at 5 ug/ml
6. Further controls included staining of cells expressing higher levels of epitope in question, and using the immunizing peptide (1 mg/ml) at a 1:50 dilution to compete out the reactivity of the antibody, resulting in a negative stain.

C. Deparaffinization:

---Label slides with date and reagent to be applied and take to the fume hood where the deparaffinization will occur

----BMRUAD= briefly move rack up and down. Move slide holder up and down so that the slides get “washed” before leaving the slide rack in the dish for the specified amount of time.

1. Dip into Xylene (in green tub in the fume hood) ---BMRUAD and then leave for 10 minutes, and repeat two more times
2. Dip into 100% alcohol, BMRUAD and then leave for 5 minutes, and repeat one more time
3. Dip into 95% alcohol, BMRUAD and then leave for 5 minutes, and repeat one more time
4. Dip into 70% alcohol, BMRUAD and then leave for 5 minutes, and repeat one more time
5. Dip into TBST buffer, BMRUAD and then leave for 5 minutes, and repeat two more times

TBST= Tris buffered saline with 0.05% Tween 20

May also use PBS pH 7.1 but TBST helps to disperse the reagent evenly over entire section

D. Antigen Retrieval **AR (should use the steamer because the microwave method is patented)**

1. fill green tub with AR buffer of choice
2. fill slide holder with “dummy” slides and intersperse the test slides among the dummy slides so that the slide holder is full of slides—this allows even heating
3. cover and place in larger container with water that goes halfway up the tub, and heat for 5 minutes on high.
4. check levels and repeat a second time, for 5 more minutes
5. remove slide container from microwave, (Careful—IT WILL BE HOT) remove lid and let the container sit on bench top for 20 minutes, to cool
6. Dip into TBST buffer, BMRUAD and then leave for 5 minutes, Repeat two more times

E. Block ENDOGENOUS PEROXIDASES. Immerse slides in slide holder in 0.3% H₂O₂ in PBS for 30 minutes at room temperature, followed with 3 buffer washes

F. Block ENDOGENOUS BIOTIN

1. Overlay with 0.1% avidin for 10 – 15 minutes (following manufacturer's suggestions), followed by 3 buffer washes
2. Overlay with 0.01% biotin for 10 – 15 minutes (following manufacturer's suggestions), followed by 3 buffer washes

G. IMMUNOSTAINING:

1. Overlay sections with CSA kit blocking solution to block non-specific protein binding
2. Overlay sections with Reagents 1 (Primary antibody used at predetermined dilution) or negative reagent controls) and incubate in humidified chamber for the specified length of time. Do not let the sections dry out at any time or this will contribute to high background.

Follow Kit instructions

N.B. Buffer has to have an increased concentration of salt, please refer to kit instructions.

MATERIALS:

1. BSA—Sigma Cat. No. A2153
2. CSA Kit—DAKO Cat. No. K1500
3. or if you prefer fluorescence, TSA kit from NEN
4. green staining dish (VWR Cat.# 25608-904)
5. white staining dish VWR Cat.# 25608-906
6. slide holder (VWR Cat# 25608-868)
7. PBS buffer phosphate buffered saline pH 7.4
8. TBS buffer : a. Biogenex Cat. # HK098-5K
b. DAKO Cat.# S1968
9. Tween 20 (Polyoxyethylene-Sorbitan Monolaureate)—Sigma Cat. No. P1379
10. Commercially available Antigen retrieval buffer from Biogenex or from Dako
11. OR Prepare 0.01 M Citrate Buffer, pH 6.0 (41 mL 0.1 M Sodium Citrate, 9 mL 0.1 M Citric Acid + 450 mL MQ Water) in 350 W Microwave – 5 min, twice see procedure outlined above
12. Avidin/Biotin Blocking Kits: a. Vector Cat. No. SP-2001
b. Dako Cat. No. X0590
c. Sigma Avidin Cat no A 9275
d. Sigma Biotin Cat no B4501
13. Mouse Anti Human Vimentin—DAKO Cat. No. N1521 prediluted
14. Mouse IgG monoclonal supernatant from P3X63 Ag8, usually at 5 ug/ml, negative reagent control
15. Rabbit anti human vWf Dako Cat # N1505
16. Rabbit IgG Dako Cat.# X0936 negative rabbit reagent control, use at 5 ug/ml
17. AEC substrate: a. Vector Cat. No. SK-4200
b. Biogenex Cat # HK092-5K
18. 30% H₂O₂—Fisher Cat. No. H325-100
19. Aquamount—Fisher Cat. No. BM-01
20. Mayer's Hematoxylin— Sigma Cat. No. MH532-1L