Performing IHC Using A Leica Linear Stainer To Deparaffinize And Rehydrate Paraffin EmbeddedTissue Slices

Rebecca Hines-Boykin, Brent Eason, Laura Markowitz, and Veena Chavakula

Describes the program for the **Leica Linear Stainer** to deparaffinize and rehydrate paraffin embedded tissue and how to do an IHC protocol on tissue following this procedure including antigen retrieval using Retrivagen A (Fisher, BD550524, BD Biosciences). Primary and secondary antibody binding and staining, counter-staining and dehydration are also described in this protocol. Total time is two partial days.

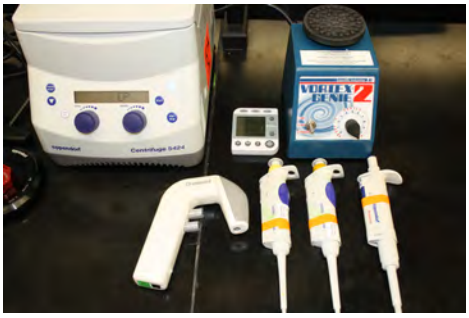
Performing IHC Using A Leica Linear Stainer To Deparaffinize And Rehydrate Paraffin Embedded Tissue Slices

NOTES: Each tissue type (i.e. mouse tumor, human tumor, what virus is being queried and other factors) requires specific antibodies and substrates for the project being run and must be optimized to find the concentration and timing most useful for the tissue being analyzed. The Appendix contains how to make buffers and other necessary reagents. The linear stainer does not allow varying times at each station in one protocol.

Immunohistochemistry:

Equipment needed-

1. Pipette aid (Fisher 13-681-15D, Drummond electric)
2. Vortex Genie 2 (Fisher has many to choose from)
3. Microwave (Any with variable power setting levels)
4. Minicentrifuge (Fisher 99-22850)
5. Pipettor, 2-20 ul (Fisher 21-377-817 or 13-690-027, Eppendorf)
6. Pipettor, 20-200 ul (Fisher 21-377-820 or 21-231-371-10, Eppendorf)
7. Pipettor, 100-1000 ul (Fisher 21-377-821 or 21-371-13, Eppendorf)
8. Timer (Fisher 06-662-55)
9. Vacuum line (in house is fine)
10. Water line with Whatman 0.45 um Polycap filter [to connect to stainer] (Fisher 97-441-00 and appropriate tubing, Fisher 14-169-7D and adapters)



Supplies needed-

1. Gloves (any nitrile or powder free latex)
2. Forceps (Fisher S17328C)
3. Slide boxes (Fisher 03-446)
4. 15 ml conical centrifuge tubes (Fisher 05-538-59A, Corning)
5. 50 ml conical centrifuge tubes (Fisher 07-203-510, Corning)
6. Microscope slides (Fisher 12-550-143)
7. Cover slips (Fisher 22-899-093, 22-899-091 or 67-763-07)
8. Slide moisture incubation trays (Fisher 684-32A)
9. Aspirator flask, 1000 ml (Fisher 10-181-7D)
10. No. 8 stopper with hole (Fisher 14-135M)
11. Aspirator tubing (Fisher 14-169-7D)
12. Barrier Pap pen (Fisher 23-769-300)
13. Plastic Coplin staining jars (Fisher 19-4)
14. 1 liter reagent bottles (Fisher 06-414-1D, Pyrex)
15. 500 ml reagent bottles (Fisher 06-414-1C, Pyrex)
16. Permanent markers (Fisher 13-379-4)
17. Labeling tape (Fisher has many to choose from)
18. 200 ul pipette tips (Fisher 02-681-147)
19. 1000 ul pipette tips (Fisher 02-681-169)
20. 5, 10 and 25 ml pipettes (Fisher 12-565-738, 12-565-739, 12-565-740)
21. 1.7 ml snap cap microcentrifuge tubes (Fisher 98-197-18)
22. Kimwipes (Fisher 06-666A)
23. Paper towels (Fisher 06-666-32B)

Reagents needed-

1. 100% Ethanol, 200 proof (Fisher BP2818-4)
2. 95% Ethanol, 190 proof (Fisher 04-355-226 or Sigma E7148)
3. Histochoice (Sigma H2779-1L)
4. TBS 1X (Calbiochem 524750- 10tabs)
5. 30% Hydrogen Peroxide (Sigma H1009-500ml)
6. Cytoseal XYL (Fisher TA-060UG, 60 ml or TA-125UG, 125 ml, Lab Vision)
7. Clear nail polish (Wet n Wild clear nail protector; sold at many stores)
8. Retrievagen A (Fisher BD550524, BD Biosciences)
9. ImmPress reagent kit, anti-mouse Ig, secondary antibody (Vector MP-7402)
10. ImmPact Nova Red 120ml (Vector SK-4805)
11. AKT Phos (Leica AKT-PHOS-L-CE)
12. Bovine serum albumin (Fisher BP1600-100)
13. Gelatin, from cold water fish skin (Sigma G7041-100G)
14. Tween 20 (Fisher BP337-500)
15. Triton X-100 (Fisher MT X 15681)
16. Normal horse serum (Vector S-2000)
17. Hematoxylin Solution, Gill's No. 3 (Sigma GHS 3-100 ml, 500 ml); Mayer's Hematoxylin Stock Solution (Fisher 50-270-48, 500 ml)
18. Ammonium Hydroxide (Fisher A669-500)

Deparaffinization/Rehydration Staining Protocol (Robot)

Program Settings: "*Dips*" – **01**; "*Start at*" – **1**; "*Stain Time*" – **200**

Solution Set-Up:

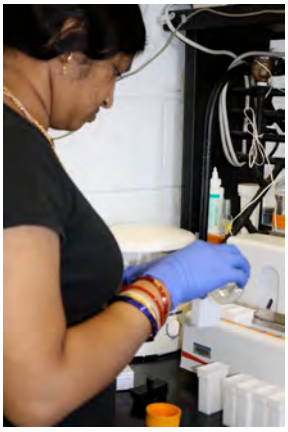
Staining Container	Solution	Amount of Time(s)
1	Histochoice	200
2	Histochoice	200
3	Histochoice	200
4	Histochoice	200
5	100% Ethanol	200
6	95% Ethanol	200
7	80% Ethanol	200
8	70% Ethanol	200
9	50% Ethanol	200
10	ddH2O (still)	200
11	ddH2O (still)	200
12	ddH2O (flowing)	(or by hand)
13	ddH2O (flowing)	(or by hand)
14	ddH2O (flowing)	(or by hand)

Protocol:

DAY 1

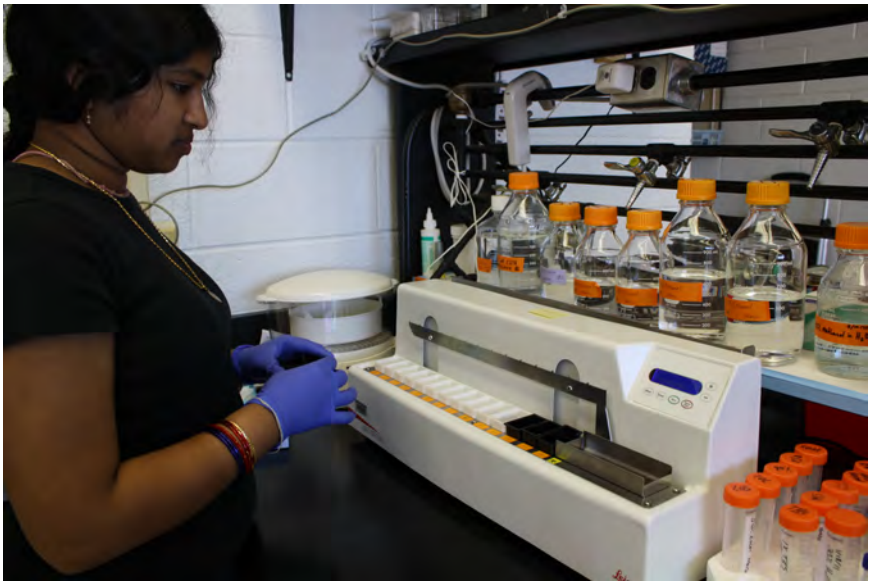
1. Ensure settings for the machine are correct (starts on page 3).
2. Place up to 4 slides in slide holders and insert one holder into the container labeled "1". (NOTE: when it moves to container 2, place another in position 1 and so forth.)



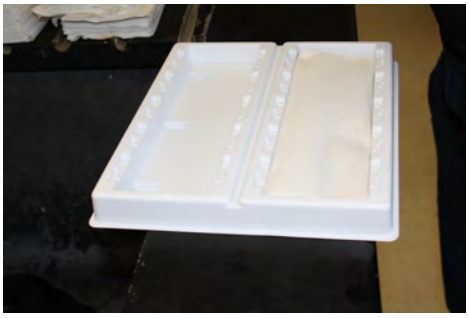


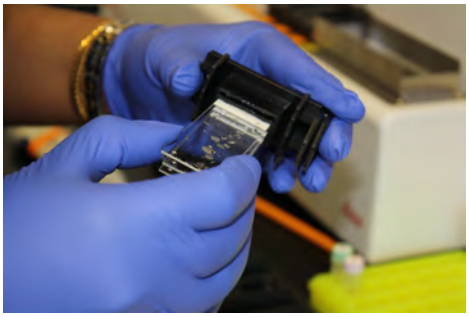

3. Press "**Run**" and allow machine to go through the programmed sequence.

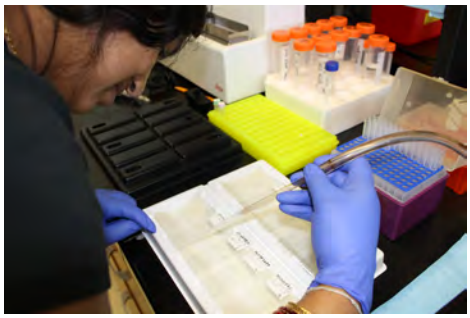
- Note: The machine will re-dip the slides every 20 seconds, eliminating the need to swish by hand.



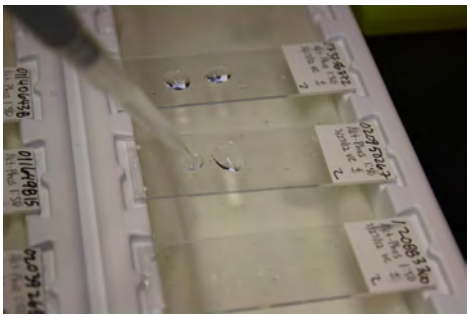
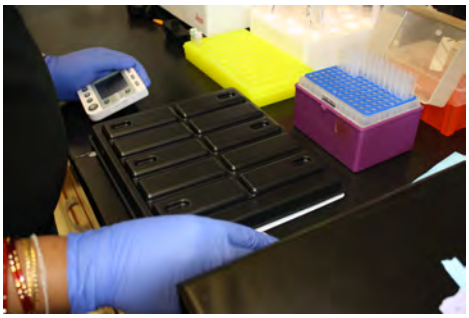
4. The machine will finish container 14, and then it will move the slides to the slide rack and beep to signal it has completed the program. (NOTE: if batch processing, you can move slide holders to a slide container filled with Nanopure or molecular water and swish by hand.)



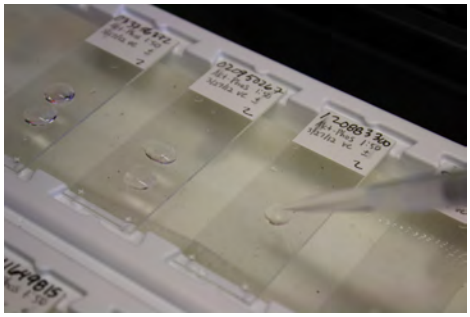


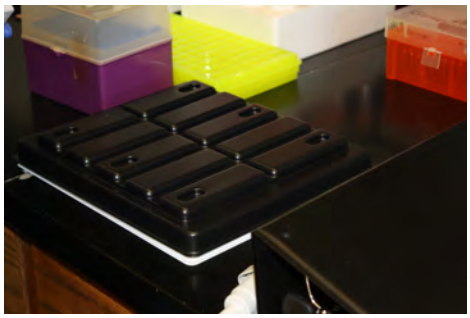


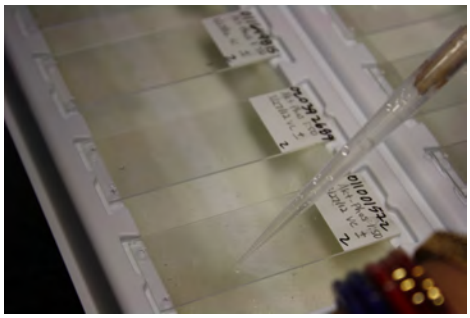
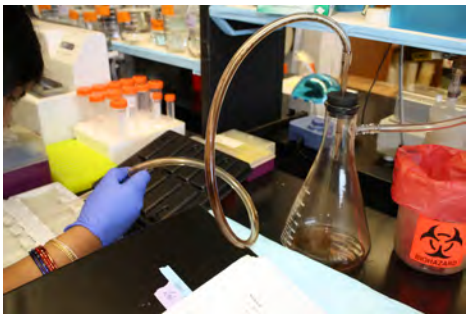
5. Place slides in 3% Hydrogen Peroxide (H_2O_2; 30% diluted with 1X TBS = 1:10 dilution) for 10 min (NOTE: H_2O_2 dilution must be made fresh daily).

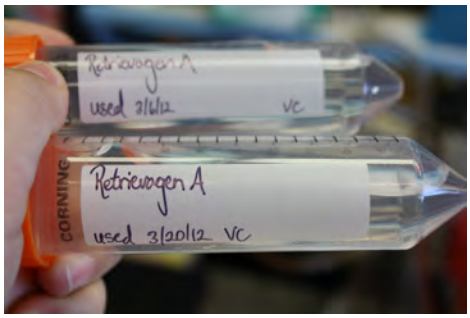

6. Wash slides in 1X TBS 2 X 3 minutes in humidity tray, aspirating between and at end of washes. Use just enough TBS to cover tissue sections, being careful not to touch tissue during aspiration.

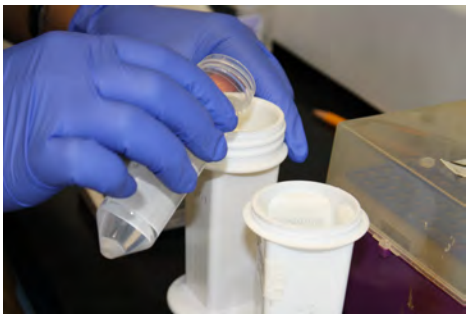



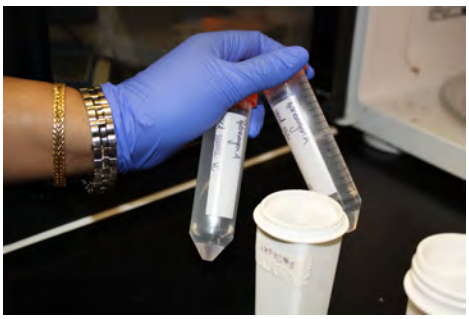
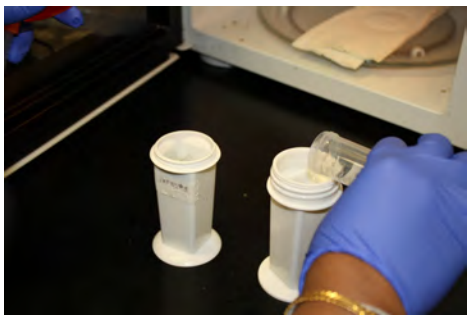


7. Place slides in appropriate containers with Retrievagen A (use a plastic Coplin jar) and run microwave for 5 min at level 2 (after coming to boil), 5 min at level 2 again, 10 minutes at level 1 adding Retrievagen A as needed (microwave procedure). Cool at room temperature for 20 minutes.

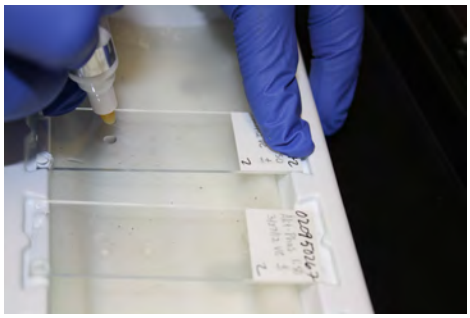


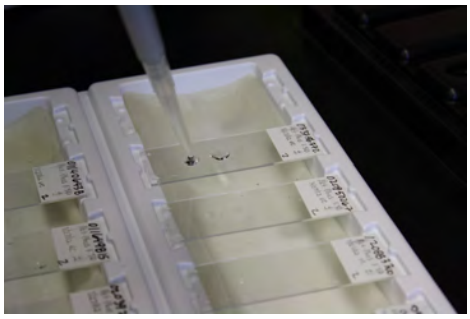



8. Wash slides in 1X TBS, 2 X 3 minutes in humidity tray (cover tissue sections), aspirating between and at end of washes.

   (Refer to step six)

9. Define outer edge of tissue with a pap pen. Add blocking serum solution (1ml 1% gelatin solution, 1 ml 10% BSA solution, 0.01 ml Triton X-100, 0.005ml Tween-20, 0.15 ml normal horse serum, dilute to 10 ml in 1X TBS). Block 30 min at RT in humidity slide tray.

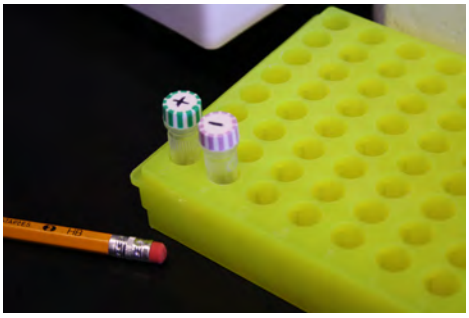
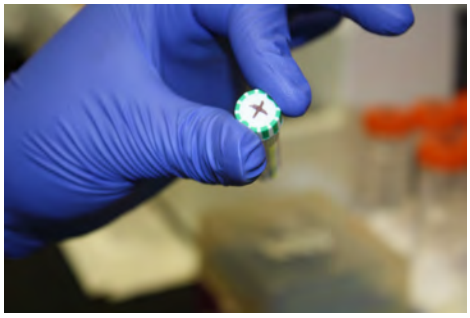


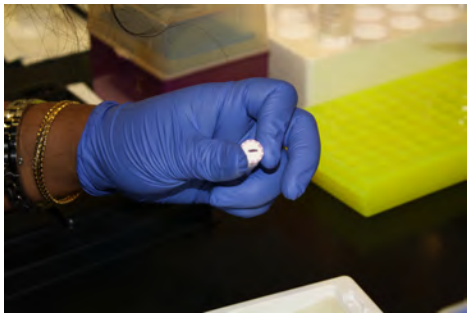


10. Wash slides in 1X TBS, 3 X 3 minutes using humidity slide tray; aspirate between washes and at end.

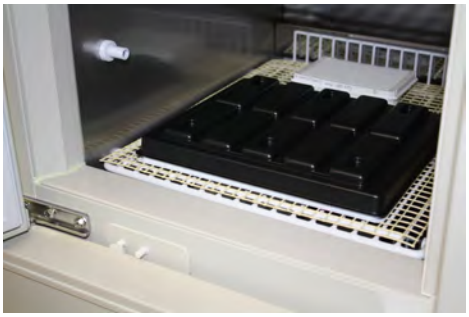
(Refer to step 6)

11. ANTIBODY STAINING STEP: Apply primary antibody (optimize concentrations for each antibody 1:50-1:500 prior to deciding which is best dilution) to positive tissues, apply antibody **diluent** to negative control tissue cuts (Used AKT-Phos, 1:50 diluted in antibody diluent). Make only the necessary amount fresh for each protocol run. (+= Antibody & Diluent and - = Diluent only)



12. Incubate overnight in humidity slide tray at 4°C.



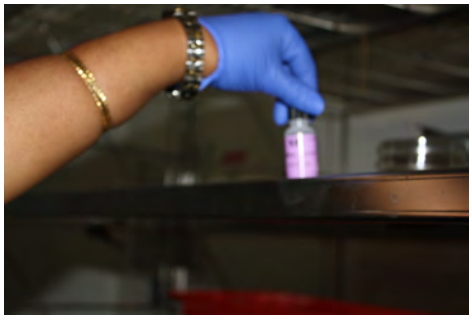
BREAK

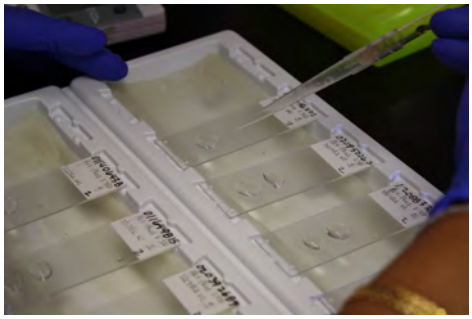
DAY 2

13. Wash 3X 3 min with 1X TBS. Aspirate between washes and at end.

(Refer to step 6)

14. Warm secondary antibody for 20 minutes in warm room (Vector ImmPress anti –mouse IgG, according to manufacturer instructions) and put on slide tissues. Incubate 30 min at RT in humidity slide tray.

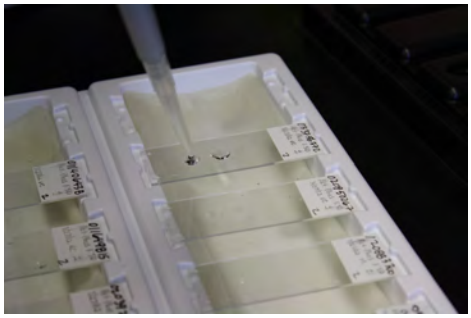




15. Rinse slides 3X 3min with 1XTBS.

(Refer to step 6)

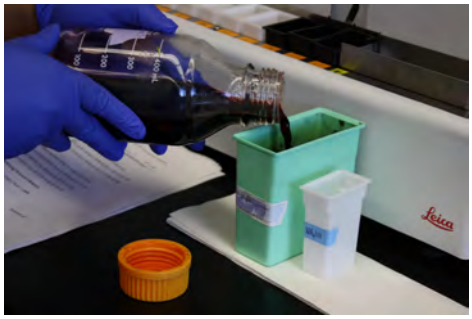
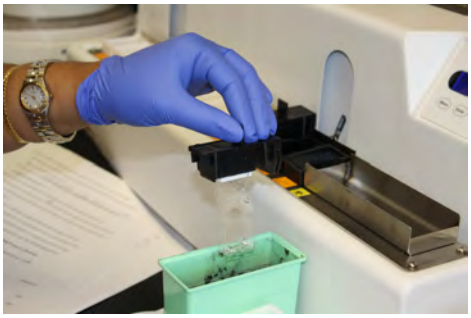
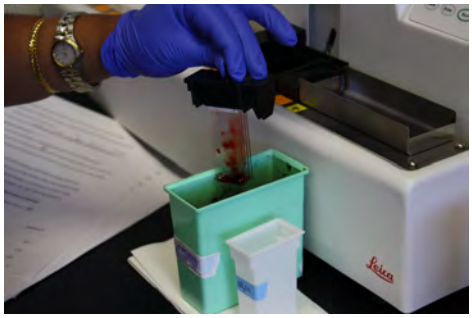
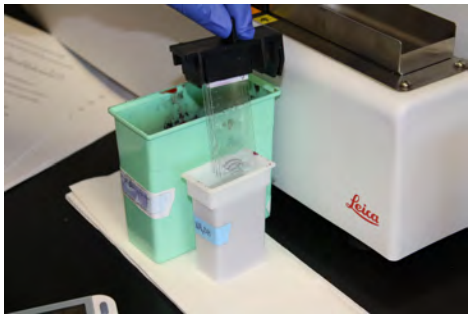
16. Prepare NovaRED solution and develop slides with the substrate: Vector ImmPact NovaRED peroxidase substrate for tumors for 15 min at RT.



17. Wash slides in 1X TBS 3 X 5 minutes, using slide tray; aspirate between washes and at end.

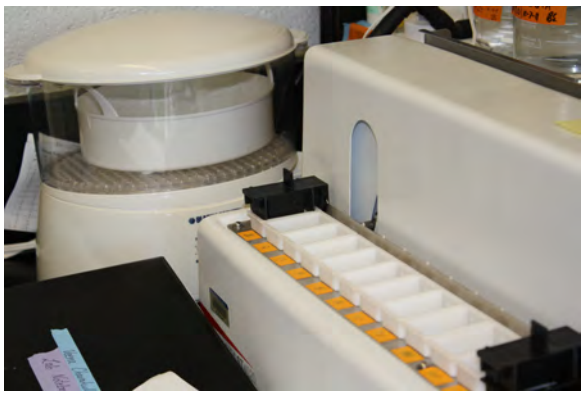
(Refer to step 6)

18. **COUNTERSTAIN** with Mayer's Hematoxylin Stock Solution for 20 seconds using a slide holder and container; dip in 1:100 Ammonium Hydroxide 10 times in container that a slide holder rack will fit in.

19. Wash in water for 5 minutes dipping frequently to wash off excess counterstain.

20. Run slides through Leica Linear Stainer, to dehydrate and fix, using Histochoice to remove Pap pen smear, per the following protocol. NOTE the containers are in a different order.



Dehydration Staining Protocol (Robot)

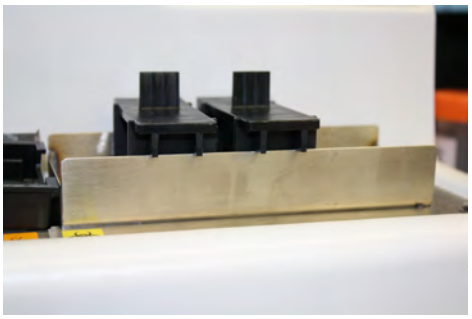
Program Settings: "*Dips*" – **01**; "*Start at*" – **5**; "*Stain Time*" – **200**

Solution Set-Up:

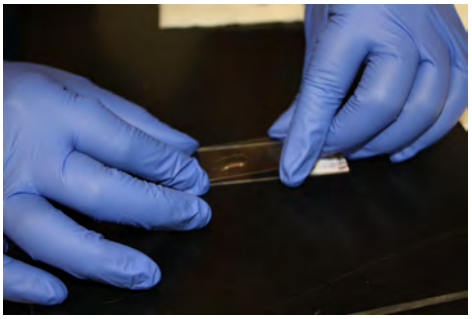
Staining Container	Solution	Amount of Time(s)
1	50% Ethanol	200
2	70% Ethanol	200
3	80% Ethanol	200
4	95% Ethanol	200
5	100% Ethanol	200
6	Histochoice	200
7	Histochoice	200
8	Histochoice	200
9	Histochoice	200

21. At end of this Dehydration Protocol, set slide rack holders in the end metal drying tray to drip/air dry.

a. NOTE: Can use Kim Wipe to wipe back side of slide only, but can gently blot tissue side with clean, doubled Kim Wipe without any rubbing or movement of it. This expedites the drying process so that the slides can be mounted and sealed sooner. DO NOT wash off the Histochoice.



22. When dry, mount coverslip using Cytoseal XYL to mount, try to avoid bubbles, allow to dry; apply clear nail protectant (Wet N Wild) to edges of coverslip to seal permanently.



Appendix of Reagents:

Dilution of 30% Hydrogen Peroxide (SIGMA 31642):

1 ml 30% hydrogen peroxide, 9 ml TBS (final concentration of 3%); may cut volumes in half if not needing 10 ml (make fresh daily)

Serum blocking solution, 10 ml, 1.5% normal horse serum:

1 ml of 1% Gelatin solution (store stock at 4 degrees)
1 ml of 10 % BSA (store stock at 4 degrees) 0.01 ml Triton X-100 (store stock at RT)
0.005 ml; (5 ul) Tween-20 (store stock at RT)
0.15ml (150 ul) Normal horse serum (store at 4 degrees)
Dilute to 10 ml with 1x TBS
(Store blocking solution at 4 degrees for up to 3 weeks)

Antibody diluent solution, store at 4 degrees:

1 ml 1% Gelatin solution
1 ml 10% BSA solution
8 ml 1X TBS
(Store stock at 4°C for up to 3 weeks)

10% Bovine serum albumin:

1 gram BSA in 10 ml dd-water
Mix 1 minute
(Store at 4° degrees for up to 2 weeks)

1% Gelatin –Cold water fish skin:

.1 gram gelatin from cold water fish skin
10 ml dd-water
mix several minutes
(Store at 4° degrees for up to one month)

1x TBS (Calbiochem 524750-10 tabs):

Dissolve 1 tablet in 500 ml dd-water
(Store at RT)

Retrievagen A:

18 ml Retrievagen A solution 1
82 ml Retrievagen A Solution 2
Bring volume up to 1 liter with dd-water
(Store at 4° degrees for up to one week)

ImmPACT NovaRED:

Preparation of Substrate Working Solution:
To 5 ml of ImmPACT Nova RED Diluent:
Add 3 drops of ImmPACT NovaRED Reagent 1
Add 2 drops of ImmPACT NovaRED Reagent 2
Add 2 drops of ImmPACT NovaRED Reagent 3
Add 2 drops of ImmPACT NovaRED Reagent 4
Stores up to 14 days at 4° C

Ethanol dilutions:

100% (200 proof, no dilution)
95% (190 proof, no dilution)
80%: 20 ml dd-water, 80 ml 200 proof ethanol
70%: 30 ml dd-water, 70 ml 200 proof ethanol
50%: 50 ml dd-water, 50 ml 200 proof ethanol

Use full strength from manufacturer:

Histochoice
Hematoxylin Stock Solution