· **TITLE: Tissue Preparation**

· **PURPOSE:**

Complete guide to Tissue Preparation (processing, embedding, cutting, staining…) and operation of Automated machines.

· **CONTENTS:**

A. Tissue processing
B. Tissue Embedding Techniques
C. Microtome / Sectioning
D. H&E staining (Leica multistainer)
E. H&E staining (Manual)
F. Reagent labeling
G. Cassettes labeler
H. Slides labeler

A. TISSUE PROCESSING:

1. DEFINITION : Tissue processing: The aim of tissue processing is to embed the tissue in a solid medium firm enough to support the tissue and give it sufficient rigidity to enable thin sections to be cut, and yet soft enough not to damage the knife or tissue.

Tissue processing stages involve:

1.1. Fixation: Fixation is the foundation for the subsequent stages in the preparation of tissue sections, up to the making of diagnosis. Most tissues are fixed before they are examined microscopically, therefore, it is essential that fixation is effective and that the appropriate fixative is used. The process of autolysis and bacterial attack should be prevented. Tissues should not change shape or volume and they should be left in a condition which subsequently allows clear staining of sections. At the same time, tissues should be as close to their living state as possible without loss or rearrangement. The choice of fixative for routine histology varies/differs for the different groups of chemical substances found in tissues. The most commonly used fixative in histopathology is 10% neutral buffered formalin. Bouin's fluid is used for fixing very delicate tissues, e.g. testis. The time required for correct, adequate and complete fixation varies from 6-48 hours depending on the size, type or nature of the tissue.

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1.2. Dehydration: To remove fixative and water from the tissues and replacing them with dehydrating fluid.
1.3. Clearing: Replacing the dehydrating fluid with a fluid that is totally miscible with both the dehydrating fluid and the embedding medium.
1.4. Impregnation: Replacing the clearing agent with the embedding medium.

2. PROCEDURE :

2.1. SAFETY

2.1.1. Caution when handling reagents.
2.1.2. Use disposable Nitrile gloves when handling carcinogens or toxic materials.
2.1.3. Do not smoke, eat or d rink in areas where specimens or reagents are handled.
2.1.4. Avoid contact of reagents with eyes and or mucous membranes. If contact with sensitive areas, wash with water.
2.1.5. Patient's specimens should be handled as biohazard materials and disposed of with Precautions.
2.1.6. Review the Safety Data Sheet (SDS) before handling reagents and solutions.
2.1.7. Each open automated tissue processor is operated at least 5 feet from the storage of combustible materials and from the paraffin dispenser

2.2. QUALITY CONTROL

2.2.1. Perform regular maintenance. (Lab.Med.-FRM-HIST 17)
2.2.2. Replace all reagents every 2 weeks with fresh reagent.
2.2.3. Check the solutions and paraffin level, if needed add it.

2.3. EQUIPMENT

2.3.1. Tissue-Tek® VIP 5
2.3.2. Tissue-Tek® VIP 6

2.4. OPERATING INSTRUCTION FOR TISSUE-TEK® VIP 5

2.4.1. After gross description arrange the tissue cassettes neatly in the tissue processing baskets. Do not pack tightly. (for details please refer to IPP-HIST-006 Criteria for Macroscopic Examination).
2.4.2. Open the retort lid, Place the processing basket in the tissue processor. Close and lock the retort lid.
2.4.3. Press button TISSUE PROCESSING to access the Start Tissue Processing screen.

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2.4.4. Select the required Program.
2.4.5. Program # 1 (To be used daily, from Sunday-Thursday)
2.4.6. Program # 2 (To be use on weekends)
2.4.7. Next screen shows a reminder for Solution change, Press EXIT to continue.
2.4.8. Select DELAY START.
2.4.9. Press STARTS key. Start confirmation Menu with Program details screen displays.
2.4.10. After all above steps performed make Shure that you have selected the right program.
2.4.11. When processing is finished the specimens are held in the final wax and the displays show Tissue Processing is completed. Press DRAIN button.
2.4.12. Then display shows "Remove basket".
2.4.13. Open the retort lid and remove the specimen basket.
2.4.14. CLEAN RETORT with cotton gauze or tissue papers.
2.4.15. Place the empty basket into the retort, close & lock the retort lid.
2.4.16. Up on closing the retort cleaning cycle screen displays.
2.4.17. Select the desired cleaning program CLEANING CYCLE-1
2.4.18. After cleaning cycles gives alarm to notify that cleaning cycle is finished.

2.5. OPERATING INSTRUCTION FOR TISSUE-TEK® VIP 6

2.5.1. Place the cassettes into the basket. Place the basket lid on the basket and lock the lid.
2.5.2. Open the retort lid
2.5.3. Place the basket into the retort, using the basket carrier, Close & lock the retort lid.
2.5.4. From the main (Process Monitor) screen, touch the Log On button. The Enter Password dialog box displays.
2.5.5. Use the touch keypad to enter your password, and then touch the Enter button.
2.5.6. The password is 123.
2.5.7. If your login is successful: The Process Monitor screen displays. Touch the Tissue Processing button to access the Start Tissue Processing screen. Select required program.
2.5.8. Start Mode - allows the user to choose to start processing in the Immediate or Delayed Start Mode - Touch Modify to choose either Immediate or Delayed Start.
2.5.9. Press STARTS key.
2.5.10. Start confirmation Menu with Program details screen displays.

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2.5.11. TO START PROCESS: Press START key.
2.5.12. Once required program is started the Machine check for vacuum pressure and reagent availability.
2.5.13. To add on more tissue(s) to the existing program. Do the following:
2.5.14. Touch Log on.
2.5.15. Enter password 123.
2.5.16. Once password enters the pause option appears.
2.5.17. Select pause and open the retort lid.
2.5.18. Add tissue(s) and close the retort, press resume.
2.5.19. When processing is finished the specimens are held in the final wax and the displays show Tissue Processing is completed. Press EXIT button.
2.5.20. Then display shows "Remove basket". Touch the Remove Baskets button.
2.5.21. The Basket Removal confirmation screen displays.
2.5.22. Open the retort lid and remove the specimen basket, using the basket carrier & tray.
2.5.23. Place the empty basket into the retort, close & lock the retort lid.
2.5.24. Touch **Yes** if all of the baskets have been removed from the retort.
2.5.25. After a processing run the **"Clean Cycle Start"** Screen displays.
2.5.26. The Clean Cycle Start Screen consists of three tabs, clean Retort, Warm Water Flush and Rinse Cycle. When a program is selected the lamp icon illuminates.
2.5.27. Touch **Start** to begin the selected clean cycle. The Clean Start Confirmation Screen displays Touch **Yes** to start.
2.5.28. Touch the **Exit** button to close the window. The Clean Retort Overview Monitor screen displays.
2.5.29. Touch **Cancel** to exit to the process menu.
2.5.30. Touch **Log off** to log out the system. When the system is in the log off condition, the Log On button will display.
2.5.31. Follow the daily / weekly maintenance procedure.

2.6. LIMITATION OF THE PROCEDURE

2.6.1. Prolonged fixation in 10% neutral buffered formalin causes shrinkage and hardening of the tissues and severely inhibit enzyme activity.
2.6.2. Poorly fixed specimens will stain poorly with routine and special stains.
2.6.3. Fixative needs around 1 hour to penetrate 1mm of tissue.
2.6.4. Fatty tissues need longer time to fix.

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2.6.5. Over – fixed specimens may give false negative results with immunohistochemistry.

2.7. TISSUE PROCESSING PROGRAMS :

2.7.1. To achieve acceptable results for diagnostic purposes, processing programs may be needed for different sizes and types of specimens. Biopsy specimens may be processed on a shorter schedule than larger specimens; large, dense or fatty specimens and brain specimens will not process adequately on a shorter schedule. A variety of processing programs should be used to achieve good processing results.

2.8. VALIDATION

2.8.1. Initial validation must be done. Except in cases if there is machine failure the validation should be repeated.

2.8.2. New tissue processing schedules must be validated against the standard laboratory processing schedule, document the record of validation by approval from Consultant Pathologist.

2.8.3. To validate new processing programs, laboratories should run tissue samples of the same size, thickness and fixation in duplicate. Reagents on the processor should be comparable, example all fresh reagents. Process, embed cut, and stain slides at the same time and evaluate the quality of the blocks, example firmness, and ease of cutting. The slides should be evaluated by the pathologist without knowledge of which processing program was used and graded on quality of section and staining. The new processing program must be equal or better quality before being put into use.

2.8.4. This method may also be used to verify a routine processing before putting a new processor into production.

Sample Table for Program # 1 / Routine / Weekend

For Large Specimen Overnight Processing

Station	Set	Solution	OC	P/V	Mix
1	2:00	10% Neutral Buffered Formalin	40	On	Slow
2.	2:00	10% Neutral Buffered Formalin	40	On	Slow
3	0.30	H2O	40	On	Slow

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4.	1:00	Alcohol 70%	40	On	Slow
5.	1:00	Alcohol 90%	40	On	Slow
6.	1:00	Alcohol 100%	40	On	Slow
7	1:00	Alcohol 100%	40	On	Slow
8.	1:00	Alcohol + Xylene 50:50	40	On	Slow
9.	1:00	Xylene	40	On	Slow
10.	0.30	Xylene	40	On	Slow
11.	0.30	Paraffin	60	Off	Slow
12.	0.30	Paraffin	60	Off	Slow
13.	0.30	Paraffin	60	Off	Slow
14.	0.30	Paraffin	60	Off	Slow
Total Time:13:00 Stirrer Speed 10 Delay Station 1		End Time(Days/Time) Level Upper Delay Heat + Stir			

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Sample Table for Program # 2 Routine For Small biopsies

Station	Duration Minute/Hour	Solution	Temp.	P/V	Mix
1	0:30	10% Buffered Formalin	40	On	Slow
2.	0:30	10% Buffered Formalin	40	On	Slow
3	0:10	Water	40	On	Slow
4.	0:15	Alcohol 70%	40	On	Slow
5.	0:15	Alcohol 90%	40	On	Slow
6.	0:15	Absolute Alcohol	40	On	Slow
7	0:15	Absolute Alcohol	40	On	Slow
8.	0:15	Alcohol + Xylene 50:50	40	On	Slow
9.	0:20	Xylene	40	On	Slow
10.	0:20	Xylene	40	On	Slow
11.	0.20	Paraffin	60	Off	Slow
12.	0.20	Paraffin	60	Off	Slow
13.	0.20	Paraffin	60	Off	Slow
14.	0.20	Paraffin	60	Off	Slow
Total Time:5:00 hour					

B. TISSUE EMBEDDING TECHNIQUES

1. DEFINITION :

1.1. Processed tissue specimens are embedded in molten paraffin wax the next morning using the tissue embedding machine.

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1.2. Cassette holders and labeled plastic cassettes are used to produce a solidified paraffin block of the specimen.

2. POLICY :

2.1. Most laboratories use an embedding center to deal with the routine workload. This comprises three modules: wax dispenser, cold plate, and a heated storage area for molds. These centers are designed to be used with the various sized mold accept the base of the cassette which will act as the object holder in the chuck of the microtome. With the Embedding machine, paraffin wax is dispensed automatically from a nozzle into a suitably sized mold, which is then placed on a small cool area to allow the wax at the base of the mold to semi-congeal. This will allow easy orientation of the cassette is placed on top and together they are placed on the cold plate so the paraffin wax can cool quickly, this ensuring a small crystalline structure. After the paraffin wax has solidified (usually after 5 minutes), the mold is removed and the block is ready for sectioning.

3. PROCEDURE:

3.1. Reagents: Paraffin wax

3.2. Equipment

· Tissue-Tek Tec 5

· Thermo Histostar

3.3. Supplies

· Tissue Cassettes

· Metal molds

3.4. Special Safety Precautions

3.4.1. Wear gloves during embedding.

3.4.2. Avoid contact of wax with eyes and mucous membranes.

3.4.3. Do not use Xylene for cleaning purposes.

3.5. QUALITY CONTROL

3.5.1. Wipe off forceps between specimens.

3.5.2. Make sure that the metal mold is free of any tissue.

3.6. OPERATING INSTRUCTIONS FOR TISSUE-TEK EMBEDDING MACHINE.

3.6.1. Turn on cold plate – Turn ON cryo key Condenser-cooling fan will run, cold plate starts to cool.

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3.6.2. Other temperatures like-paraffin tank, metal mold storage area, and other are preset.
3.6.3. Turn "On" orientation platform lights.
3.6.4. Operating temperatures hot area: 60 C° - 70 C°.
3.6.5. Place processed cassettes in the holding chamber to keep paraffin melted.
3.6.6. Turn "off" "Cold plate only" after use.
3.6.7. Refer to Tissue-Tek operator's manual for further instructions.
3.6.8. Daily cleaning has to be done

3.7. OPERATING INSTRUCTION FOR THERMO HISTOSTAR.
3.7.1. The cold plate is ON automatically every day except on weekends, if further needed it can be turned on by touching cooling option from the main LCD screen menu.
3.7.2. Open the wax dispenser knob anti clock wise to dispense wax.
3.7.3. The light automatically turns on while using wax dispensing lever.
3.7.4. Cold plate should be switched off when not in use to prevent excessive frost build up. Other than the cold plate wax tank, forceps chamber, base mold compartment and tissue storage tank are auto set temperature.
3.7.5. Refer to Thermo Histostar user's manual for detailed instructions.

Temperature control

Paraffin Reservoir ºC Increments ± 1 °C	+45 - +70	+58 ºC
Mold warmer Increments °C	+35 - +60 ºC ± 1	+60 ºC
Cassette bath Increments	+45 - +70 ºC ± 1 °C	+58 ºC
Work area ºC	+45 - +70	+62 ºC

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Increments	± 1 °C	
Cold plate and Cold spot	-5 ºC	-5 ºC
Forceps holder	+70 ºC	+70 ºC
Paraffin dispenser + pump	+45 - +70 ºC	+58 ºC

3.8. EMBEDDING TISSUES:

3.8.1. Cassettes are removed from the paraffin wax.

3.8.2. The plastic lid is removed from one cassette at a time.

3.8.3. The suitable mold is chosen.

3.8.4. Tissue is picked out from cassettes by a pair of forceps.

3.8.5. Tissue is placed in the mold.

3.8.6. The side of the tissue from which it is desired to take sections is placed face down.

3.8.7. All other tissues must be carefully oriented so that the plane of sectioning will be correct e.g. Walls of cysts must be embedded edge down and biopsies of skin surface must be embedded so that the plane of skin surface is vertical to the bottom of the mold.

3.8.8. The tissue specimen is pressed down in the mold for a few seconds until the cooling wax holds it.

3.8.9. Forceps are kept clean between specimens to prevent cross contamination.

3.9. PROPER TISSUE PLACEMENT DURING EMBEDDING PROCEDURE.

3.9.1. It is very important to know the proper embedding for each tissue.

3.9.2. Flat tissue section – Embed on flat cut surface. Examples: Ovary, breast tissue, lymph node, uterine sections (cervix and uterine body), tonsils, placenta, lipoma, thyroid, keloid, tip of appendix.

3.9.3. Cut surface – Embed on cut surface of tissue section. Example: Bisected skin ellipse, hemorrhoids.

3.9.4. Segmental section – Embed on flat surface of segment. Examples: Tubular tissues with a lumen, such as fallopian tubes, vas deferens, varicose veins, appendix, umbilical cords.

3.9.5. Section of tissue with large lumen – Embed on cut surface to demonstrate Lining. Examples: Intestines, foreskin, hernia sac, ganglion cyst, gallbladder, pterygia.

3.9.6. Curettage biopsy – Embed fragment longitudinally to see as much of tissue as possible. Examples: Uterine scrapping, prostate chips.

3.9.7. Circular of horseshoe shaped punch biopsy – Embed on end to show horseshoe shape. Example: Cervical biopsy, bladder

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biopsy, biopsy of GI tract.
3.9.8. Larger polypoid lesion – Embed on long axis to show plane of cut section. Example: GI and endometrial polypoid lesion.
3.9.9. Resection with surgical margins indicated by Indian ink – Embed on side with ink. Example: Skin excision from Breast Carcinoma or Melanoma

3.10. METHOD LIMITATIONS
3.10.1. Embedding media is paraffin wax only.

C. MICROTOME / SECTIONING:

1. DEFINITION :

1.1. Embedded paraffin blocks are trimmed to expose the whole surface of the tissue using a microtome. After cooling the immersed surface, thin sections of 2-4 microns (um) are sectioned. (Some tissues for special stains e.g. Congo red stain may require thicker sections). Floated out in a water bath, picked unto a glass slide, labeled with the Pathology number of each specimen and allowed to drain and dry. After this, place the slide on a hot plate at about 45 ºC to sufficiently melt off the paraffin and at the same time enable the section to adhere to the slide after which the slide is stained routinely for H&E or by special stains.
1.2. PRINCIPLE: Rotary microtomes, when maintained and used properly, are capable of cutting sections from paraffin blocks as thin as 1µm. The rotary action of the handle (via the internal mechanism) causes the head of the microtome to which the paraffin block is secured, to advance by the number of microns set on the section thickness dial. As the block passes through the blade, a section will be produced according to this thickness setting. Once the sections are placed on a water bath, the expansion of wax due to surface tension and heat helps to remove wrinkles and folds. In addition to a functioning microtome, sharp and blemish-free blades are essential for satisfactory cutting. Poorly prepared paraffin blocks may be sectioned with a good blade, but a poor blade may fail to cut even the best material.

2. Policy:

2.1. All staffs performing microtomy are responsible for keeping their own work area clean and free of paraffin, ensuring that the microtome, waterbath and work area are left tidy at the end of each day and that disposable blade holders and blades are stored so as not to be potentially dangerous. At the end of each week, in addition to leaving the work area clean and tidy, the user must lubricate the microtome according to the lubrication points denoted on the instrument. Routine Hematoxylin & Eosin (H&E) sections

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will be prepared on regular slides (uncoated). Sections for other staining procedures will be prepared on positively charged (coated) slides so the sections will not fall off during the staining procedure. The required number of slides and stains must be prepared in a timely manner to facilitate and diagnosis

3. PROCEDURE:

3.1. Safety: Microtome blades are extremely sharp and carelessness can lead to serious injury. Blade holders from which the blade has been removed may be left in the microtome.

3.1.1. When inserting blocks into or removing blocks from the microtome head, the microtome flywheel (to which the handle is attached) must be locked in place so that it will not rotate.

3.1.2. Disposable blades must be removed from the holder if the microtome is left unattended for extended periods of time.

3.1.3. Used blades must be disposed of in appropriately identified sharps containers.

3.1.4. For long-term storage or transport, keep the blade holder in the manufacturer's box.

3.1.5. Make sure blade-holder boxes are securely closed before lifting up from the bench or out of a drawer.

3.1.6. Never lay a blade-holder down on the bench or other open surface, where it is likely to cause injury.

3.1.7. Never run or walk around with a blade in your hand.

3.1.8. If a blade or blade holder is dropped, never attempt to catch it. Allow it to land before retrieving.

3.1.9. Keep fingers away from the blades. Use forceps, brush or a probe to retrieve sections from the blade.

3.1.10. All injuries must be reported to the Histopathology supervisor.

3.2. Equipment and Materials

- Rotary microtome
- Blade-holder
- Float bath
- Cold Plate
- Hot Plate
- Disposable blades
- Paraffin blocks for sectioning

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· Glass slides

· Probe, Forceps, Brush, Pencil

3.3. Quality Control

3.3.1. Change the blade if visible scratch / lines along the section are seen, and nicks on the blade edge.

3.3.2. To avoid contamination/ carryover, change daily the water in the float bath.

3.4. Sectioning

3.4.1. Assemble all materials and set up the water bath. The float bath should be heated to Approximately 45°C. This will vary according to individual preference.

Note: Having the temperature too high may cause 'cracking' artifact due to rapid expansion of the sections, particularly lymphoid tissue.

3.4.2. Insert a paraffin block into the block clamp.

3.4.3. Unlock blade holder to advance the paraffin block face to the blade-edge then lock it.

3.4.4. Turn the small crank located on the left side of the microtome against clockwise one quarter to bring the block back from the blade-edge.

3.4.5. Remove excess paraffin to expose the entire tissue surface for subsequent thin sections by turning the main crank located on the right side of the microtome continuously clockwise and the paraffin block will move against the knife.

3.4.6. Do not trim away too much tissue.

3.4.7. Put the block face down on the cold plate to make paraffin firm enough to be easily sectioned.

3.4.8. Clean the Microtome with a soft brush.

3.4.9. Insert the block into the chuck; bring it into line with the knife.

3.4.10. Section thickness is 2-3 microns for routine Hematoxylin and eosin staining, 3 microns for imunohistochemistry or as mentioned in related policies. For special tissue please see the table below.

Tissue	Thickness
Routine Paraffin	**2 to 4 microns**
Renal Sections	**1 to 3 microns**
Bone marrow	**2 to 3 microns**
Nerve histochemical staining	**6 to 15 microns**

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Amyloid demonstrating	6 to 12 microns

3.4.11. Crank wheel is turned with the right hand continuously until sections are seen on the knife. They often come off the block in a thin ribbon and should be the same shape and size as the face of the block.
3.4.12. Hold the ribbon carefully onto the water bath.
3.4.13. Immerse one slide to three-quarters of its length.
3.4.14. Maneuver the section strip using the slide so that sections stick to the slide.
3.4.15. Section residue should be cleaned from water bath after each block (specimen) to prevent the carry over.
3.4.16. Label the slide according to the block data with a pencil. Do not label slide first. Incorrect pre-labeled slides may accidentally be picked up and used.
3.4.17. Allow the slides to stand vertically in a rack to air dry.
3.4.18. Transfer the slides to a staining rack and placed in a hot air oven at 60°C - 64 °C for 30 minutes until wax is melted to proceed for H&E staining.

3.5. Notes / Tips

3.5.1. It's a common practice amongst most of the technologist that spreading of sections with 70% alcohol prior to transferring them to the water-bath facilitates the removal of folds and/or wrinkles.
3.5.2. For tissues prone to lifting off or for lengthy or arduous staining procedures, coated (positively charged) slides should be used, e.g. hard or decalcified tissues, blood clot, etc.
3.5.3. If tissue is calcified, soak in decalcify solution for 5 – 10 minutes depending on the level of calcification. Prolonged soaking may cause the tissue to swell and dislodge from the paraffin block.
3.6. The slides prepared as described above, then stained by the hematoxylin and eosin stain (refer to IPP 14.HIST 06B) then submitted to the pathologist on duty, along with the patient's pathology report form request, for microscopic examination and evaluation/ diagnostic interpretation after screening

3.7. Slide requirements and Quality

3.7.1. Serial sections: Sequential sections obtained from the block.
3.7.2. Levels / Deeper: Sections taken at 10µm intervals unless otherwise stated.
3.7.3. Biopsies: 3 H&E slides (levels) cut at 3µm.
3.7.4. Any other tissues: 1 slide for H&E and further cuts on requests by the pathologists.
3.7.5. Referral Paraffin block: 1 H&E slide cut at 3 µm from each block.

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3.8. Histopathology slides must be of adequate technical quality to be diagnostically useful. Criteria to evaluate include adequate tissue fixation, processing, thickness of sections, absence of interfering tissue folds and tears, and good staining technique and cover slipping. For Hematoxylin and Eosin and other routine stains, the patient slide serves as the internal control to ensure adequate staining technique. The sections must be cut from sufficient depth in the block to include the entire tissue plane. Document the Quality of slides for every day run on Lab.Med.FRM-HIST 011 (Quality of slides Log sheet)
3.9. The pathologist will decide whether special stains and/or immunohistochemistry are needed for further diagnostic interpretation. Requests for special stain and immuno-histochemistry slides shall cut on further request from the pathologist. (Example, deeper, levels, etc.).
3.10. Microtome Maintenance :
3.10.1. Section waste should be removed with a dry brush. Any mild detergent may be used for cleaning.
3.10.2. The microtome can also be cleaned with alcohol or xylene. Disinfectant can also be used after cleaning.
3.10.3. All moving parts should be lubricated once a month. This includes the clamping levers and their shafts screws, slide ways of the knife holder, base, sliding surface between knife holder bases.
3.10.4. Document on Lab.Med.FRM-HIST 016

3.11. Float Bath Maintenance
3.11.1. Use a soft brush to remove any air bubbles that may form in the bottom and sides of the float bath chamber.
3.11.2. Change the water daily or as the need arises to prevent cross-contamination of paraffin Sections.
3.11.3. Blot the water surface with soft tissue to prevent the so called “floaters or extraneous Tissues”.
3.11.4. Document on Lab.Med.FRM-HIST 002.

D. HEMATOXYLIN AND EOSIN STAIN (Leica Multi-stainer)

1. DEFINITION: The Hematoxylin and Eosin stain is the most commonly used stain in Histology. It is the basis of most diagnosis and the starting point for all other investigation. Hematoxylin - stain the nucleus blue-purple. Eosin - stain the cytoplasm and connective tissue element. Together these two stains make the best general morphological stain in use. Leica Multistainer detects program by various color clips according to the color assigned to the program.

2. PROCEDURE :

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2.1. SAFETY

2.1.1. Numerous chemicals and dyes are used in staining procedures. Persons performing staining procedures must take every precaution to protect themselves. Appropriate Personal Equipment (PPE) and clothing are essential. All spills must be cleaned up immediately and appropriately according to SDS sheets.

2.2. SPECMEN

Paraffin embedded tissue fixed in 10% neutral buffered formalin. Cut paraffin section at 2-4um.

2.3. CONTROL

2.3.1. Control slide tissue (example, Appendix, Colon) to be run daily before any other slides are stain.
2.3.2. Control slide must be documented on H&E QC worksheet (Lab.Med.FRM-HIST 011).

2.4. SOLUTIONS

2.4.1. Hematoxylin (Harris and Gill)
2.4.2. Eosin Solution 0.2% (Aqueous base)
2.4.3. 1% Acid Alcohol
2.4.4. Ammonia Water
2.4.5. Isopropanol (follow safety Guidelines while handling).
2.4.6. Xylene (follow safety Guidelines while handling).

2.5. MATERIALS

2.5.1. Slide Racks from Lecia Multistainer
2.5.2. Multi-Color Clips from Leica Multistainer

2.6. OPERATING INSTRUCTIONS

2.6.1. Turn on power switch to initialize the instrument.
2.6.2. Select the appropriate slide rack with desired color clip.
2.6.3. Insert slide correctly in the slide rack (specimen side of the slide must face forward towards the word).
2.6.4. Open the loading door in front of the instrument.

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2.6.5. Load the Slide Rack to Loading Station.
2.6.6. Close the loading door.
2.6.7. The machine identifies the program and Robotic arm Picks up the slide Rack
2.6.8. Automatically.
2.6.9. Do not disturb or touch the Robotic arm while its moving.
2.6.10. After finishing the program it drops the rack at Unloading Station.

2.7. STAINING PROGRAM - COLOR CLIP **BLUE**

Station #	Solutions	Program H&E Routine
1	Xylene	5 minutes
2	Xylene	5 minutes
3	Xylene	5minutes
4	Absolute Alcohol	10 DIPS
5	Absolute Alcohol	10 DIPS
6	Absolute Alcohol	10 DIPS
7	Absolute Alcohol	10 DIPS
8	Running Water	1 minute
20	Hematoxylin	9 minutes-15second
9	Running Water	2 minutes
14	1% Acid Alcohol	2 dips
10	Running Water	2 minutes
15	Bluing solution (Ammonia water)	30 seconds
10	Running Water	1 minutes
21	Eosin Solution	2 minuts15 seconds
13	Absolute Alcohol	10 dips
17	Absolute Alcohol	10 dips

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18	Absolute Alcohol	10 dips
19	Absolute Alcohol	10 dips
16	Xylene	15 seconds
22	Xylene	3 minutes
23	Xylene	3 minutes

2.8. RESULTS:

2.8.1. Nuclei Blue to blue black
2.8.2. Cytoplasm Pink
2.8.3. Calcified bone Purplish blue
2.8.4. Collagen Light pink
2.8.5. Muscle fibers Deep pink

2.9. NOTES :

2.9.1. Staining times will need to be increased towards the end of the week as the stains age and their staining ability lessens.
2.9.2. Tissue blocks that have been subjected to prolonged decalcification prior to processing may need to have an extended staining time in hematoxylin and a decreased staining time in eosin.

2.10. QUALITY CONTROL

2.10.1. Every day, a quality control H&E stain must be performed on control block processed the same way as the rest of the specimen blocks.
2.10.2. If the H&E QC stain is deemed acceptable, the staining times used during it, will be used. If the quality of the QC H&E is not acceptable, the staining times must be adjusted accordingly.
2.10.3. Also, the technical quality of histologic preparation is assessed by the pathologist on a daily basis. This is documented on the Histology Quality Control sheet (Lab.Med.FRM-HIST 011).
2.10.4. All solutions in the automatic stainer are changed weekly, filtered daily (hemtoxylin) and documented in form (Lab.Med.FRM-HIST 029)

2.11. QUALITY OF SLIDES

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2.11.1. Histopathology slides must be of adequate technical quality to be diagnostically useful.
2.11.2. For normal routine work 76x25 mm slides are universally used with preferred thickness 1.0-1.2 mm (because they do not break easily).
2.11.3. Ground and polished edges will significantly reduce the danger of finger cuts.
2.11.4. Frost ended slides, either double or single, is the fastest and equally secure method to use.
2.11.5. Absence of interfering tissue folds/ tears and air bubbles.
2.11.6. Air bubbles or spaces around the tissue should be avoided.
2.11.7. Sections must be cut from sufficient depth in the block showing the entire tissue plane, cut 2-4 microns.

2.12. LEICA 5030 ROBOTIC COVERSLIPPER
2.12.1. Leica CV5030 is a robotic cover slipper for cover slipping tissue sections mounted on slides with cover slips.
2.12.2. Supplies And Materials **:**
· D.P.X media
· Xylene
· Mountant bottle with lid
· Plastic / steel slide rack
· Coverslip catcher
· Dispenser nozzle, 18 G
· Container for prime-solution
· Suction cups
· Coverslips (24x50) prefer

2.12.3. Activate power switch to initialize the instrument.
2.12.4. If prime is shown on the display, prime must be carried out first.
2.12.5. Transfer the dispenser at working position.
2.12.6. Place the rack arranged with slides on the bath.
2.12.7. Press Stat.
2.12.8. After finishing the slide press Lift to get the slide rack up.
2.12.9. Once the coverslipping is done remove the rack from the bath.
2.12.10. Refer to operator manual for complete instruction.

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2.12.11. QUALITY CONTROL

· Avoid air bubbles by slowly filling the mountant bottle with mountant.
· Select desired coverslip of high quality (<0.17mm) that will cover the whole tissue preparation.
· Replace damaged or deformed suction cups as needed.
· Clean dispenser needle from any mounting fluids after every processed rack.
· Use only recommended mounting medium with high viscosity that will protect the tissue/slide from damage and dust, to preserve the cell characteristic and quality of the stain and allow a better microscopic examination under various magnifications.

E. Hematoxylin and Eosin by Manual staining

1. DEFINITION :

1.1. Hematoxylin is extracted from the heartwood ("logwood") of the tree Hematoxylin campechianum which originated in the Mexican state of Campeche, but is now mainly cultivated in the West Indies. The hematoxylin is extracted form logwood with hot water, and then precipitated out from the aqueous solution using urea (Lamb, personal communication 1974). It is important to realize that hematoxylin itself is not a stain; it is its oxidation product, hematein, which is the natural dye. Hematein can be produced from hematoxylin in two ways:

1.2. **Natural Oxidation ("ripening")** by exposure to light and air. This is a slow process, sometimes taking as long as 3-4months, but the resultant solution seems to retain its staining ability for a long time. Ehrlich's and Delafield's hematoxylin solutions are examples of naturally ripened hematoxylins.

1.3. **Chemical Oxidation:** using sodium iodate (e.g. Mayer's Hematoxylin) or Mercuric oxide (e.g. Harris's Hematoxylin). The use of chemical oxidizing agents converts the hematoxylin to hematein almost instantaneously, so these hematoxylin solutions are ready for use immediately after preparation. In general, they have a shorter useful life than the naturally oxidized haematoxylins, probably because the continuing oxidation process in air and light eventually destroys much of the hematein, converting it to a colourless compound.

1.4. Haematein is anionic, having a poor affinity for tissue, and is inadequate as a nuclear stain without the presence of a mordant. The most useful mordants for haematoxylin are salts of aluminum, iron and tungsten, although haematoxylin solutions using lead as a mordant are occasionally used (for example in the demonstration of Angyrophil cells). The mordant/metal cation confers a net positive charge to the dye-mordant complex and enables it to bind to anionic tissue sites such as nuclear chromatin. The type of mordant used influences very strongly the type of tissue components stained and their final color. Most mordents are incorporated

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into the haematoxylin staining solutions, although certain Haematoxylin stains required the tissue section to be pretreated with the mordant before staining; an example is Heidenhain's iron hematoxylin.

1.5. PRINICPLE: The Haematoxylin and Eosin stain is probably the most widely used histological stain. It is the basis of most diagnosis and the starting point for all other investigations. Most histological preparations are routinely stained with haematoxylin and eosin. Its popularity is based on its comparative simplicity and ability to demonstrate clearly an enormous number of different tissue structures. Haematoxylin can be in numerous ways and has a widespread applicability to tissues from different sites. Essentially, the haematoxylin component stains the nuclei blue/black, with good intranuclear detail, whilst the eosin stains cell cytoplasm and most connective tissue fibers in varying shades and intensities of pink, orange and red. However, haematoxylin has many more uses than in the haematoxylin and eosin combination.

2. PROCEDURE :

2.1. SAFETY

2.1.1. Numerous chemicals and dyes are used in staining procedures. Persons performing staining procedures must take every precaution to protect themselves. Appropriate Personal Equipment (PPE) and clothing are essential.

2.1.2. All spills must be cleaned up immediately and appropriately according to MSDS sheets. Wear gloves and mask when changing solutions.

2.2. SPECIMEN

2.2.1. Paraffin embedded tissue fixed in 10% neutral buffered formalin. Cut paraffin section at 2-4um.

2.3. CONTROL

2.3.1. Control slide tissue (example, Appendix,Colon) to be run daily before any other slides are stain. Control slide must be documented on H&E QC worksheet (Lab.Med.FRM-HIST 011).

2.4. SOLUTIONS

2.4.1. Hematoxylin

2.4.2. Eosin solution 0.2 % (Aqueous base)

2.4.3. 1% Acid Alcohol

2.4.4. Ammonia water

2.4.5. Alcohol graded (follow safety guidelines while handling).

2.4.6. Xylene (follow safety Guidelines while handling).

2.4.7. Slide racks.

2.5. STAINING

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2.5.1. Slides are removed from the oven into xylene, a process called de-waxing as follows
· Xylene – 5 minutes each three (3) changes.
· Graded Alcohol – 5 to 6 dips in each.
· Wash well in running water.
2.5.2. Hematoxylin 5 – 10 minutes.
2.5.3. Wash in running water.
2.5.4. Differentiate in 1% Acid Alcohol
2.5.5. Wash in running water 3-4 minutes for bluing.
2.5.6. Eosin 2-5 minutes.
2.5.7. Rinse in tap water.
2.5.8. Dehydrate in graded Alcohol 4 changes 2-3 dips in each.
2.5.9. Clear in Xylene 3 changes 10 dips in each.
2.5.10. Mount with D.P.X media.
2.5.11. Leica Robotic cover slipper can be use for mounting.

2.6. **RESULTS**
2.6.1. Nuclei---------- Blue to blue black
2.6.2. Cytoplasm---------- Pink
2.6.3. Calcified Bone---------- Purplish blue
2.6.4. Collagen ---------- Light pink
2.6.5. Muscle fibers---------- Deep pink

2.7. **NOTE**: Staining times will need to be increased towards the end of the week as the stains age and their staining ability lessens.

F. REAGENT LABELING.

1. POLICY :

All persons preparing reagents and solutions must follow the established methods outlined below. All working solutions, reagents and stains must be properly labeled with the contents, and if applicable, date they are changed / filtered and expiration date.

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2. PROCEDURE :

2.1. SAFETY.

Numerous chemicals and dyes are used in preparing solutions for routine histology and for staining procedures. All persons making up these solutions must take every precaution to protect themselves. Appropriate Personal Protective Equipment (PPE) and clothing are assential (gloves, cryo gloves, facemask, vapor mask, goggles, face shield). All spills must be cleaned up immediately and appropriately according to Material Safety Data Sheet. For more information on each chemical, refer to its MSDS.

2.2. EQUIPMENTS AND MATERIALS.

· Analytical balance.
· Magnetic stirrer.
· Measuring cylinders.

2.3. PROCEDURE FOR MANUFACTURER PREPARED REAGENTS.

2.3.1. Note the date that the reagent was received.
2.3.2. Store according to the manufacturer's instructions and rotate stock accordingly.
2.3.3. Upon opening, read the package insert for manufacturer's instructions and recommendations, note the date of opening and before use, perform the necessary QC tests to demonstrate satisfactory performance of the reagents.
2.3.4. Document prepared solution information on Solution Preparation Log Book.

2.4. PROCEDURE FOR LABORATORY PREPARED REAGENTS.

2.4.1. Prepare the reagent according to policy.
2.4.2. Label with the reagents name, date of preparation, expiration date and initial.
2.4.3. Document the reagent preparation on the QC sheet (not done any more, commercially prepared).
2.4.4. QC the reagent with a known positive control (if applicable, e.g. staining solutions.
2.4.5. Store appropriately.

2.5. ALCOHOL SOLUTIONS.

2.5.1. 95% Alcohol
· Absolute Alcohol ---------- 3800 ml
· Distilled water---------- 200 ml

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2.5.2. 90% Alcohol
· Absolute Alcohol ---------- 3600 ml
· Distilled water ---------- 400 ml

2.5.3. 70% Alcohol
· Absolute Alcohol---------- 2800 ml
· Distilled water ---------- 1200 ml

2.6. BOUIN'S FIXATIVE (shelf life – One year at R/T). Commercially prepared.
· Picric acid, saturated aqueous solution---------- 750 ml
· Formalin 37% w/v----------250 ml
· Acetic acid glacial---------- 50 ml

2.7. 10% NEUTRAL BUFFERED FORMALIN (shelf life – One year at R/T). Commercially prepared.
· 40% Formalin (Formaldehydes)---------- 2.0 L
· Distilled water---------- 18.0L
· Sodium Dihydrogen Phosphate (anhydrous)---------- 80 gm
· Disodium hydrogen phosphate---------- 130 gm
This solution has a pH of approximately 6.8-7.4 and it is hypotonic in the buffer ions present (RETIRED)

2.8. GLUTARALDEHYDE (shelf life – 6 month at 4° C).
· 50% Glutaraldehydes----------1.0ml
· Phosphate buffered saline----------30ml

2.9. EVALUATION OF EXPIRED REAGENTS AND REAGENTS LACKING MANUFACTURER EXPIRATION DATE.
2.9.1. The Histopathology laboratory's Safety Officer assigns expiration date to any reagents that do not have a manufacturer-provided expiration date (with a maximum of 5 years). The assigned expiration date should be based on known stability, frequency of use, storage conditions, and risk contamination.
2.9.2. This policy applies to all reagents used in the laboratory (Histochemical, Immunohistochemical and Immunofluorescent

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reagents).
2.9.3. The acceptable performance of histochemical stain is determined by technical assessment on actual case material, use of suitable control sections, (know positive controls) and as part of the pathologists diagnostic evaluation of a surgical pathology cases.
2.9.4. A document has to be maintained all the time whenever an expired solution/reagent used. LAB.MED-FORM GEN 023, Lab.Med-FRM-HIST 037
2.9.5. Expired Reagents May Be Used Only Under The Following Circumstances:
2.9.5.1. The reagents are unique, rare or difficult to obtain.
2.9.5.2. Delivery of new shipments of reagents is delayed through causes not under control of the laboratory.
2.9.5.3. The laboratory must document validation of the performance of expired reagents in accordance with written laboratory procedure.

G. CASSETTE LABELER (IPC) LEICA

1. POLICY :

1.1. All laboratory personnel designated to operate the Leica IPC must read operating manual carefully and must be familiar with all technical features of the instrument.
1.2. Operator is responsible for daily and weekly maintenance.
1.3. Once the machine indicates for replacing ink inform to supervisor.

2. PROCEDURE :

2.1. SAFETY

2.1.1. Potentially lethal voltages are present inside the instruments. Do not remove panel or access covers.

2.2. QUALITY CONTROL

2.2.1. The serial surgical pathology number and medical record number is labeled on cassettes according to the policy. (Refer to

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IPP 14.HIST 03)
2.2.2. Perform regular maintenance on (Lab.Med-FRM-HIST 042)
2.2.3. Before cassette labeler in use, must be validated. See below for validation procedure.
2.2.4. The imprint field of imprinted cassettes should never be touched or wiped while damp.
2.2.5. Take care when removing excess paraffin from cassettes. Scraping may damage the imprint field, making the print illegible.

2.3. OPERATING INSTRUCTION
2.3.1. Switch on the cassette labeler from the rear side button only for one time, unless the machine is not to be use for long time. Also turn on the computer connected with Leica IPC.
2.3.2. Fill all the magazines with suitable tissue cassettes.
2.3.3. Load cassettes trays on to the automated unload station and press "tray load".
2.3.4. Press "online" button to wake up instrument.
2.3.5. Go to computer and select the shortcut icons (KFHU,Astoon,Referral etc.)
2.3.6. Fill the required data and print.
2.3.7. If any error messages or flash refer to the troubleshoot guide which is place above the instrument.
2.3.8. Perform cleaning of head weekly. (Follow the instructions from the user's manual which is placed above the instrument).

2.4. VALIDATION
2.4.1. Initial validation must be done. Except in cases if there is change in the cassette brand, validation should be repeated.
2.4.2. Print multiple cassettes and run the tissue processing programs used in the histopathology lab to ensure that the ink is resistant against the various reagents the cassette will subsequently be exposed to.
2.5. MAINTENANCE
2.5.1. Perform daily and weekly maintenance document on Lab.Med-FRM-HIST 042.
2.5.2. Once a week the print head must be cleaned, follow the user's manual instruction.

H. SLIDE LABELER (IPS) LEICA

1. POLICY :

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1.1. All laboratory personnel designated to operate the Leica IPS must read operating manual carefully and must be familiar with all technical features of the instrument.
1.2. Operator is responsible for daily and weekly maintenance.
1.3. Once the machine indicates for replacing ink inform to supervisor.

2. PROCEDURE :

2.1. SAFETY

2.1.1. Potentially lethal voltages are present inside the instruments. Do not remove panel or access covers.
2.1.2. Do not touch the ramp during operation. Risk of injury.
2.1.3. Do not open the reflector flap of the flashlight while the instrument is ON-risk of burns and blinding.

2.2. QUALITY CONTROL

2.2.1. The serial surgical pathology number and medical record number is labeled on slides according to the policy. (Refer to IPP 14.HIST 03)
2.2.2. Perform regular maintenance on (Lab.Med-FRM-HIST 043)
2.2.3. Before slide labeler in use, must be validated. See below for validation procedure.
2.2.4. The imprint field of imprinted slides should never be touched or wiped while damp.

2.3. OPERATING INSTRUCTION

2.3.1. Switch on the slide labeler from the rear side button only for one time, unless the machine is not to be use for long time. Also turn on the computer connected with Leica IPS.
2.3.2. Fill all the magazines with clipped corner snow coat slides from Leica.
2.3.3. Load slides trays on to the automated unload station and press “tray load”.
2.3.4. Press “online” button to wake up instrument.
2.3.5. Go to computer and select the shortcut icons (KFHU,Astoon,Referral etc.)
2.3.6. Fill the required data and print.
2.3.7. If any error messages or flash refer to the troubleshoot guide which is place above the instrument.
2.3.8. Perform cleaning of head weekly. (follow the instructions from the user’s manual which is placed above the instrument).

2.4. VALIDATION

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2.4.1. Initial validation must be done.
2.4.2. Print multiple slides and run for routine staining used in the histopathology lab to ensure that the ink is resistant against the various reagents the slides will subsequently be exposed to.

2.5. MAINTENANCE

2.5.1. Perform daily and weekly maintenance document on Lab.Med-FRM-HIST 043. Once a week the print head must be cleaned, follow the user's manual instruction.

· **RESPONSIBILITIES:** It is responsibility of the technologist who operates the machine to perform maintenance according to the manufacturer guidelines.

· **ATTACHMENTS:**

· Lab.Med.FRM-HIST 002 Float bath Maintenance
· Lab.Med.FRM-HIST 005 Cold Plate Maintenance
· Lab.Med.FRM-HIST 005 Hot Plate Maintenance
· Lab.Med.FRM-HIST 047 Quality for slides log sheet
· Lab.Med.FRM-HIST 013 Cover slipper Maintenance
· Lab.Med.FRM-HIST 014 Embedding Maintenance
· Lab.Med.FRM-HIST 015 Mutlistainer Maintenance
· Lab.Med.FRM-HIST 016 Microtome Maintenance
· Lab.Med.-FRM-HIST 017 Tissue Processor Maintenance
· LAB.MED. -FORM GEN 023 Expiry extension record
· Lab.Med.FRM-HIST 029 H& E Stain Solutions QC.
· Lab.Med-FRM-HIST 037 Expiry extension record
· Lab.Med.-FRM-HIST 042 Cassette Printer Maintenance
· Lab.Med.-FRM-HIST 043 Slide Printer Maintenance

· **DISTRIBUTION:**

· LMD Administration Office
· Histopathology Laboratory Section
· DQSA

· **REFERENCES:**

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- Operation Manual Sakura Tissue-Tek VIP 5 and Sakura Tissue-Tek VIP 6
- Tissue-Tek Embedding Operation Manual.
- Thermo Histostar Operation manual.
- Reference Manual, Leica RM2235 Rotary Microtome.
- Instruction Manual of Leica ST 5020 Mutlistainer.
- Instruction Manual of Leica CV 5030 Cover slipper.
- Laboratory Safety Manual.
- Chemical Hygiene Plan.
- Operation Manual Leica IPC
- Operation Manual Leica IPS
- College of American Pathologist (CAP) Checklist.2014.

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