H&E Troubleshooting Table

Problem	Cause	Solution
1. Nuclei not crisp, "smudgy" nuclei, nuclear bubbling or no distinct chromatin pattern seen. There is no variation in the nuclear chromatin patterns among cells, and there is no variation in chromatin staining within one nucleus.	a) Fixation is incomplete. b) Fixation delayed (time to fixation was inadequate). c) Quality of fixative was poor due to solution exhaustion/overused.	i) Allow tissues to fix for a longer time. ii) Tissue should be placed in fixative (formalin) immediately. iii) Document time in fixative to assure adequate fixation. iv) Exchange the fixative in the containers (grossing table and tissue processor) at regular intervals.
	b) Water is not completely removed during the processing dehydration.	i) Ensure the dehydration is sufficient by reviewing processing schedule. ii) Ensure that processing solutions are rotated and/or exchanged on a schedule to prevent solution exhaustion.
	c) Slides were exposed to excessive heat during processing or drying.	i) Ensure that tissues are not exposed to high temperatures, and if increased temperature is required that it be as low as possible and exposure time is limited. ii) Assess whether paraffin baths provide radiant heat to the retort, causing temperature to be increased during dehydration and clearing steps. Adjust exposure times to counter increased heat. iii) Remove excess water from cut slides by standing slides on end or edge to allow water to drain thoroughly before drying. Dry slides at as low a temperature as possible.
2. Three distinct shades of eosin not seen.	a) Fixation inadequate.	i) Ensure adequate fixation by prolonging the time allowed. ii) Exchange the fixative in the containers (grossing table and tissue processor) at regular intervals.
	b) Improper processing occurred.	i) Ensure that proper processing occurs, by validating each step for completeness.
	c) There is poor differentiation of the eosin.	i) Ensure good differentiation of the eosin. Differentiation of eosin occurs best in 70% alcohol solutions and to a lesser extent in higher grade alcohols. Therefore, adding 70% alcohol to the dehydration series or increasing the time in the 70% alcohol will aid in differentiation. ii) Ensure that the 70% or 95% alcohol used in eosin differentiation is changed at regular intervals.
	d) Eosin solution is not at the correct pH. The correct pH for eosin stain is pH 4.0-4.5. Check manufacturer's SDS for pH for commercially prepared stains.	i) Exchange eosin solution on stainer with fresh eosin or adjust by adding acetic acid. ii) Ensure that slides are well rinsed in water after "bluing" to avoid carry over of alkaline solutions into the eosin stain.

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Problem	Cause	Solution
3. Poor contrast between the nuclear stain (hematoxylin) and the cytoplasmic stain (eosin).	a) The nuclear stain is too dark.	i) Decrease the intensity of the hematoxylin staining by decreasing the time in the hematoxylin solution or increasing the time in the nuclear differentiating solution. ii) Check the pH of the hematoxylin daily to maintain an optimum acidic solution (pH 2.5-2.9). Adjust the pH using the acid used in the original formulation.
	b) Nuclear stain is too pale compared to the cytoplasmic stain.	i) Increase the intensity of the hematoxylin stain by: • Increasing the time in the hematoxylin stain • Decreasing the time in the nuclear differentiating solution • Change to a stronger hematoxylin formulation ii) The pH of the water used to rinse slides after hematoxylin staining must be neutral • Acidic rinse water will act as a differentiator, removing hematoxylin • Iron in tap water can act as a hematoxylin differentiator • Water pH can be affected by agricultural runoff or other contaminants from old plumbing lines. iii) The pH of running tap water rinses can be controlled by placing ionization filters in the water line or by connecting directly to a de-ionized or distilled water source.
	c) The cytoplasmic stain is too dark compared to the nuclear stain.	i) Decrease the intensity of the eosin stain by: • Decreasing the time in the eosin stain • Diluting the eosin stain • Change the eosin stain formulation • Adjusting concentration of phloxine dye, if used • Allowing more time in the 70% or 95% dehydrating alcohols
	d) The cytoplasmic stain is too light compared to the nuclear stain.	i) Increase the intensity of the eosin stain by: • Allowing a longer time in the eosin stain • Decreasing the time in the 70% or 95% dehydrating alcohols • Ensuring the pH of the eosin is between 4.0 and 4.5 and adjust if required • Change to an eosin-phloxine formulation

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Problem	Cause	Solution
4. Cytoplasmic stain (eosin) is too dark; the cytoplasmic stain is so intense that the differentiation between collagen, smooth muscle and red cells is lost.	a) Staining time in the eosin is too long	i) Decrease the staining time in the eosin solution.
	b) There is inadequate eosin differentiation in the alcohols that follow the eosin stain.	i) Increase the differentiating time in the 70% or 95% dehydrating alcohol.
	c) Aqueous eosin formulations stain tissues darker than alcohol-based stains.	i) Switch to an alcohol-based eosin which is easier to differentiate in the graded alcohols used for dehydration.
	d) The alcohol rinse used for differentiation is not performed properly after eosin staining.	i) Change the first alcohol rinse from 100% to 95% alcohol or from 95% to 70% alcohol.
	e) Eosin may be too concentrated, especially if phloxine is present.	i) If phloxine is present, decrease the concentration or change to an eosin only formulation. ii) Dilute the eosin with diluted alcohol (70%, 95%) that is used in the original formulation.
	f) Isopropyl alcohol was used as the dehydrating alcohols; isopropyl alcohol does not differentiate eosin in the same manner as ethyl alcohol.	i) Change the type of dehydrating alcohol used. ii) Increase the time in the isopropyl alcohol rinses. iii) Use a diluted isopropyl alcohol (e.g., 95% or 80%) as the first alcohol following the eosin in dehydration, follow with the standard 99% isopropyl alcohols.
5. Cytoplasmic stain (eosin) is too light. The cytoplasmic stain is so pale that the differentiation between collagen, smooth muscle and red cells is lost.	a) The eosin solution is overused/exhausted.	i) Replace the current eosin stain with a fresh eosin stain.
	b) The eosin has passed the expiry date.	i) Replace eosin with fresh (in date) solution. ii) Rotate commercially prepared supplies to ensure oldest lot number is used first. iii) Prepare only sufficient volume of eosin to be used with established stabile date range.
	c) The staining time in the eosin stain is too short.	i) Increase the staining time in eosin solution.
	d) The pH of the eosin staining solution is greater than 4.5.	i) Check the pH of the staining solution; it should be between 4.0 and 4.5. If necessary, adjust the pH with acetic acid.
	e) Eosin stain formulation is not correct (eosin dye concentration low)	i) Adjust concentration of eosin dye in staining solution and re-validate staining method. ii) Change eosin formula to include phloxine dye and re-validate staining method. iii) Decrease alcohol dilution (add more water) in the staining solution
	f) The bluing solution (pH 8) is not completely washed out of the section or off the glass slide or staining rack before the slides are transferred to the eosin solution.	i) Increase rinse time in running water (or more changes) after bluing solution. Carryover of the bluing reagent can raise the pH of the eosin. Eosin with a pH 5 stains lighter than eosin pH 4.5.
	g) Differentiation in diluted alcohols is prolonged.	i) Decrease the time in the differentiation/dehydrating alcohols.
	h) The alcohol rinse after the eosin stain is incorrectly performed.	i) Validate that correct staining procedure is being followed.

H&E Troubleshooting Table

Problem	Cause	Solution
6. Nuclear stain too dark, the nuclear stain is so dark that the chromatin pattern is lost, and some non-nuclear elements show hematoxylin staining.	a) The hematoxylin solution is too strong due to formulation.	i) Replace hematoxylin solution with weaker formulation (ie,, replace Gills III with Gills II).
	b) Staining time in the hematoxylin stain is too long.	i) Adjust staining protocol, decreasing the staining time in hematoxylin.
	c) There is inadequate differentiation of the hematoxylin stain.	i) Adjust staining protocol, increasing time in the differentiation solution. ii) Change formulation of differentiation solution • Change concentration of acid Decrease alcohol concentration (e.g., change from 95% alcohol to 70% alcohol)
7. Nuclear stain too light, the nuclear stain is so light that well defined chromatin patterns cannot be seen.	a) Incomplete deparaffinization.	i) Increase the number of changes of xylene, or xylene substitute used for removal of paraffin. ii) Increase the amount of time in each xylene, or xylene substitute, used for paraffin removal.
	b) The hematoxylin is overused/exhausted or used beyond its shelf life.	i) Replace hematoxylin with fresh (in date) solution. ii) Rotate commercially prepared supplies to ensure oldest lot number is used first; use by expiration date provided by manufacturer. iii) Prepare only sufficient volume of hematoxylin to be used with established stabile date range. iv) Prevent overoxidation of hematoxylin solution by storing in a tightly closed container away from direct sunlight. Store in a dark container. Store at room temperature.
	c) The pH of the hematoxylin is incorrect. The correct pH for hematoxylin solution is pH 2.4-2.9. Check manufacturer's SDS for pH for commercially prepared stains.	i) Check the pH of the hematoxylin solution. If necessary, adjust the pH using the acid used in the original formulation.
	d) The hematoxylin is diluted by carryover from a previous water rinse.	i) Change to fresh hematoxylin solution. Establish a regular change schedule for reagents.
	e) Sections are over differentiated.	i) Decrease the time in the differentiating solution. ii) Decrease the acid concentration of the differentiation solution. iii) Increase the alcohol content of the differentiating solution from 70% to 95% alcohol.
	f) Staining time in hematoxylin solution is too short.	i) Increase the time in hematoxylin solution.

H&E Troubleshooting Table

Problem	Cause	Solution
7. (Cont'd)	g) Additives/contaminants are present in tap water rinses (before or after the staining).	i) The pH of the water used to rinse slides after hematoxylin staining must be neutral Acidic rinse water will act as a differentiator removing hematoxylin ii) Iron in tap water can act as a hematoxylin differentiator iii) Water pH can be affected by agricultural runoff or other contaminants from old plumbing lines. The pH and contaminants of running tap water rinses can be controlled by placing ionization filters in the water line or by connecting directly to a de-ionized or distilled water source.
	h) Poor fixation and or processing, resulting in tissues that are unable to bind the stain.	i) Ensure that tissue blocks are well fixed prior to processing and are well dehydrated, cleared and paraffin infiltrated during processing protocol.
	i) Section too thin.	i) Thin sections result in less tissue binding sites for hematoxylin to attach. Increase staining time may help only to a certain degree. Recut thicker sections.
8. Uneven hematoxylin or eosin staining; the stain varies in intensity in different areas of the section.	a) The section may be thick and/or thin; chatters or venetian blind effect.	i) Recut the section, ensuring that the section is of uniform thickness.
	b) Some solutions are not high enough to cover entire section, resulting in a distinct line across the section.	i) Ensure that all solutions are of sufficient volume to completely cover the tissue sections, especially when slide rack is only partially filled.
	c) Nuclear differentiation solution (acid alcohol) or bluing solution volume is higher than wash water levels. Acid alcohol and/or bluing reagent become trapped between slides/in slide racks and not washed away by water; they then drip down the slide causing vertical lines to appear on the slide where staining intensity is affected.	i) Ensure that water wash levels are higher than acid alcohol and bluing solution levels.
	d) The water rinse was not adequate after hematoxylin staining to remove excess hematoxylin.	i) Increase the time and/or fluid levels of water rinses.
	e) The water rinse after acid alcohol was not adequate to stop differentiation of hematoxylin.	i) Increase the time and/or fluid levels of water rinse after acid alcohol.

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Problem	Cause	Solution
9. Red brown nuclei. The nuclear stain has a distinct red brown or reddish hue, often seen throughout the entire slide.	a) The sections have not been sufficiently blued.	i) Increase the amount of time the sections remain in the bluing solution. ii) Ensure the pH of bluing solution is minimally pH 7-8. iii) Increase water rinsing time post hematoxylin differentiation.
	b) The hematoxylin is breaking down due to over oxidation of the hematein.	i) Change to a fresh solution of hematoxylin. ii) Check expiry date of hematoxylin solution.
10. Dark precipitate scattered throughout the section; blue-black or purple precipitate is present on parts of the section.	a) Deteriorated hematoxylin. Hematoxylin is used beyond the expiry date or damaged from improper storage conditions.	i) Change to a fresh solution of hematoxylin. ii) Ensure proper storage of hematoxylin solutions according to manufacturer's guidelines. iii) Check with supplier that hematoxylin was stored correctly during shipping. Excessive heat or freezing can cause solution to decompose.
	b) Some hematoxylin formulations (e.g., Harris hematoxylin) form a metallic sheen on the surface of the solution when exposed to air. This metallic sheen transfers or adheres to the surface of slide and the tissue section.	i) Monitor the hematoxylin solution throughout the day for the appearance of a metallic sheen (hematein). If this is observed, replace or filter the hematoxylin, ensuring that the solution container is clean and free of deposits before reuse.
11. Sections with an overall hazy appearance or eosin bleeding throughout the section, the tissue appears hazy or out of focus when examined microscopically.	a) Dehydrating and clearing solutions are contaminated with water from previous solutions or humidity.	i) Design and implement a standardized, routine, solution change schedule for alcohols and xylene that minimizes humidity contamination or helps keep carry over from prior solutions to a minimum.
	b) Sections were not adequately dehydrated after eosin staining.	i) Use a minimum of three changes of anhydrous alcohol at the end of the staining series ii) Increase the amount of time per station, for each anhydrous alcohol, at the end of the staining series. iii) Establish a schedule for regularly changing the anhydrous alcohols used for dehydration.
12. Mucus staining with hematoxylin.	a) Hematoxylin formulations differ in their abilities to stain acid mucin. Gill hematoxylin formulations will stain specifically acid mucins. Harris formulations due to their excess alum will not stain acid mucins.	i) Depending on the preference of the pathologist change hematoxylin formulation being used. ii) For proprietary formulations check with the manufacturer for staining characteristics of acid mucin. iii) Acid differentiation of acid mucins staining by Gill hematoxylin will decolorize both the mucin and the nuclei.

H&E Troubleshooting Table

Problem	Cause	Solution
13. Mounting artifact – air is under the coverslip and the image of the tissue is obscured or mounting media is on top of the coverslip, making focusing on some areas of the tissue difficult.	a) The mounting media has retracted from the edges of the coverslip.	i) Gently remove the coverslip by exposing the slide to xylene or xylene substitute to soften and remove mounting media. Expose section to fresh solutions of clearant and remount a new coverslip ii) Ensure the mounting media is not too thin. Xylene or toluene is used as a diluent in the mounting media and as it evaporates the resin can retract from the edges of the slide. iii) Ensure that automated or manual process is not adding or retaining too much xylene on slide surface to thin mounting media.
	b) The mounting media has not spread to the edges of the coverslip.	i) Gently remove the coverslip by exposing the slide to xylene or xylene substitute to soften and remove mounting media. Expose section to fresh solutions of clearant and remount a new coverslip. ii) Ensure that automated or manual process is applying sufficient mounting media to spread across entire surface of coverslip. The volume required must be adjusted to the size of the coverslip used. iii) Ensure that the surface of the slide holds sufficient xylene to assist with the spread of the mounting media.
	c) Air bubbles are trapped under the coverslip.	i) Gently remove the coverslip by exposing the slide to xylene or xylene substitute to soften and remove mounting media. E pose section to fresh solutions of clearant and remount a new coverslip. ii) Avoid mixing or shaking mounting media when adding to automated or manual dispensing bottle. If this occurs, wait and allow bubbles to rise to the surface and dissipate.
	d) There is mounting media on the top of the coverslip.	i) Gently remove the coverslip by exposing the slide to xylene or xylene substitute to soften and remove mounting media. Expose section to fresh solutions of clearant and remount a new coverslip.
14. Brown granular deposit, similar in appearance to formalin pigment, is seen throughout the section. The nuclei appear glossy, black, and refractile with brown stippling.	a) The tissue section has dried out before the coverslip was applied.	i) Gently remove the coverslip by exposing the slide to xylene or xylene substitute to soften and remove mounting media. Expose section to fresh solutions of clearant and remount a new coverslip.