GUIDELINES FOR HEMATOXYLIN & EOSIN STAINING

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Guidelines for Hematoxylin and Eosin Staining

The desired end result of a tissue stained with hematoxylin and eosin (H&E) is based upon what seems to be almost infinite factors. Pathologists or the diagnostician have individual preferences for section thickness, intensities, and shades. The choice of which reagents to use must take into consideration: cost, method of staining (manual vs. automatic), option of purchasing commercially-prepared or technician-prepared reagents, safety, administration policies, convenience, availability, quality, technical limitations, as well as personal preference. Realizing the various options, these guidelines are designed to assist the histotechnician in obtaining the individually preferred results consistently.

I. REAGENTS

A. **Clearants:** Xylene or Xylene Substitutes

1. **Use.** Clearants are used in two different stages in H&E staining.
   a. Deparaffinization –removal of paraffin.
      (1) **Troubleshooting.** If paraffin is not totally removed from tissue sections, color intensity may be decreased or staining may be irregular(spotty) within the tissue section.

      (2) **Recommendations for deparaffinization:** Use three changes of xylene, 3 minutes each station. If an automatic stainer is used where there are limited numbers of staining vessels, use at least 2-3 changes of xylene irrespective of time. If using xylene substitutes, follow the directions provided by the manufacturer.* Xylene substitutes are slower in action, and often require longer times or more stations than xylene.

   b. Clearing – displacement of alcohol from the tissue sections with the clearant to assure miscibility when coverslipping with xylene – toluene or other petroleum-based mounting media.

      (1) **Troubleshooting**
         a. Inadequate time in the xylene will allow anhydrous (100%) alcohol to remain within the tissue sections. The presence of alcohol may cause the eosin to bleed from the tissue section after coverslipping.

         b. Inadequate dehydration (removal of non-anhydrous (70%, 95% alcohol) from the tissue section will cause slides to be hazy or milky. Removal of non-anhydrous alcohol is <u>not</u> the role of the clearant; it is the role of anhydrous (100%) alcohol. The haziness/milkiness observed with the coverslipped slide is usually caused by the mixing of water from the alcohol with the clearant.

      (2) **Recommendations for clearing**: Use three changes of xylene, 1 minute minimum per station. If an automatic stainer is used where there is a limited number of staining vessels, use at least 2-3 changes of xylene irrespective of time. If using xylene substitutes, follow the directions provided by the manufacturer.

*Several times in these guidelines, manufacturer's directions will be cited. These guidelines are unable to provide all directions for the variety of reagents available to the histology lab. If the reagents have been sold for use in diagnostic procedures the FDA requires that written directions for proper use be included in the package.

2. **Varieties.** The grade of xylene used should be known and carefully monitored. Lower grades (technical, industrial, engineering) often contain other petroleum products that can interfere with staining. The presence of these unwanted petroleum products can be easily detected by their extraneous odor.

B. Alcohols: ethanol, denatured ethanol, isopropyl alcohol

1. **Use.** Alcohols are used in two different stages in H&E staining.
   a. Hydration – the introduction of water into the tissue section. This is done by passing the slides slowly through a series of decreasing concentrations of alcohols.

      (1) **Troubleshooting**. If the clearant is not displaced by the anhydrous alcohol, subsequently allowing carryover of the clearant into lower concentrations of alcohol, clearant droplets (oil droplets) will be visible in the lower alcohols and sometimes in the water. In extreme cases, the clearant may remain in the section and will interfere with hematoxylin staining.

      (2) **Recommendations for hydration**: The first alcohol used after the clearant must be anhydrous (100%). To prevent the carryover of the clearant into lower alcohol concentrations, three changes of anhydrous alcohol are suggested, followed by alcohols of lower concentrations: 95% (1-2 changes), 70% (1 change), and sometimes 50% (1 change). Immersion times should be sufficient to assure the complete removal of the previous solutions. One minute is adequate for each station. If an automatic stainer is used where there are limited numbers of staining vessels, use at least 2-3 changes of anhydrous alcohol irrespective of time. To further save stations it is possible to go directly from 95% alcohol into water without adverse effects; however, graded series may be needed for delicate specimens or weakly adhered sections.

   b. Dehydration – the removal of water from the tissue section.

      (1) **Troubleshooting.** Increasing concentrations of alcohol after eosin staining are used to remove water from the tissue section. If all water molecules are not removed from the tissue section, proper clearing cannot be achieved. Inadequate removal of non-anhydrous (70%, 95%) alcohol prior to entering the clearant for coverslipping will cause slides to be hazy or milky. The haziness is due to the mixing of the water in the alcohol with the clearant.

      (2) **Recommendations for dehydration:** The use of 70% or 95%alcohol after eosin is dependent upon desired eosin intensity. With or without the use of 70% or 95% alcohols, three changes of anhydrous alcohol should be used, one minute each. If an automatic stainer is used where there are limited numbers of staining vessels, use art least 2-3 changes of anhydrous alcohol irrespective of time.

2. **Varieties.** The grade and formulation of alcohol is important. Use of pure ethanol – a potential, taxable alcoholic drink – is under control of the Federal government. Depending upon the type of user, a variety of permits, taxes, and records must be administered. This burden can be extensive, and to alleviate this situation, certain denatured ethanols can be used. Denatured ethanol has additional chemicals (denaturants) added to it to make it undrinkable. The extent of government regulations with these alcohols is greatly reduced or eliminated. There are numerous formulations of denatured ethanol. Some denatured ethanols have ingredients that could interfere with dehydration, hydration, and staining. Specific symptoms can be fading of the stains or poor staining (due to the presence of denaturants that are oxidizers or reducers) and milky appearance of diluted alcohols (due to presence of denaturants that are not water soluble). If using denatured ethanol know the formulation and monitor it. The following chemicals are used to denature

ethanol and have no adverse effects on H&E staining: methanol, isopropyl alcohol, and MIBK (methyl isobutylketone).

3. **Storage.** All alcohols have the capacity to absorb moisture from the air. Humidity can interfere with proper dehydration and clearing of the slides; therefore, it may be necessary to rotate anhydrous (100%) alcohols more frequently during humid conditions. Storage of anhydrous alcohol should be in containers that allow minimal exposure to air. With a change in temperature, the moisture in the air inside the container could condense into water droplets that would contaminate the alcohol. If anhydrous alcohol is purchased in 55 gallon drums, inlet bungs should be set up with a drying tube (especially with humid conditioned or when the drum is used over a long time period) that will remove the moisture from the air before it enters the drum.

C. Hematoxylin

1. **Use.** Hematoxylin is used after deparaffinization and hydration. It stains the nucleus of the cell, specifically, the chromatin within the nucleus and the nuclear membrane. The nucleoplasm of the nucleus should remain unstained; this allows the staining pattern of the chromatin to be seen easily.

a. Troubleshooting

(1) Poor hematoxylin staining can be due to:

(a) Autolysis or poor fixation.
(b) Incomplete deparaffinization
(c) Overdecalcification
(d) Inadequate staining time
(e) Destaining – too strong or excessive time
(f) Weak hematoxylin that has lost its potency with age or carryover of water.
(g) Contaminants in rinsing solutions.
(h) Thin sections
(i) Incorrect pH of the hematoxylin
(j) Inadequate removal of alcohol or insufficient pre-rinsing with water prior to staining with hematoxylin

(2) Excessive staining of hematoxylin can be due to:

(a). Drying of tissue section.
(b). Strong potency of hematoxylin from “topping up”, change in formulations, or age
(c). Excessive staining times
(d). Excessive slide adhesive (e.g., albumin, gelatin)
(e), Destaining – too weak or inadequate time
(f). Thick section
(g), Prolonged exposure to heat

(3) Removal of metallic sheen. Except for Gill hematoxylins, all hematoxylins develop a surface metallic sheen upon standing or with use. If not removed, the metallic sheen will appear as a due precipitate on the slide. Therefore, all hematoxylins except Gill require daily filtering to remove the metallic sheen.

2. **Varieties.** There are numerous formulations of hematoxylin available. Individual preference of the pathologist or diagnostician must be a primary consideration. The active ingredient in hematoxylin solutions is hematein complexed with a metal ion-eg, aluminum, iron, tungsten. Aluminum is the most commonly used. If aluminum is used, the hematoxylin solution will stain blue; if iron is used hematoxylin will stain black or blue-black. The ratio of metal to hematein may also influence the color.

Hematein is formed by oxidizing hematoxylin. Oxidizing agents used are air, sodium iodate, mercuric oxide, and potassium permanganate. Hematoxylin formulae that use mercuric acid as an oxidizer warrant special disposal considerations because of the mercury. Over-oxidation will cause poor staining and can occur during the initial manufacturing or with aging of the hematoxylin.

The mode of hematoxylin staining can be progressive or regressive. Regressive staining overstains the tissue and then decolorizes the tissue with an acid solution. Progressive staining stains to desired intensity without
initially overstaianing. Progressive hematoxylins may be used in a regressive method. See Hematoxylin Differentiator discussion.

Progressive Hematoxylins:	Mayer's
	Delafield's
	Gill's
	Harris'
Regressive Hematoxylins:	Delafield's
	Harris'
	Ehrlich's

These formulations provide a variety of hues, potencies, and staining patterns. Some (Gill's) stain goblet cells, others (Harris') do not. Gill hematoxylins have three formulations (I, II, and III). Gill I has a strength which stains the delicate chromatin pattern in cytological preparations. Gill II and III are used in tissue staining. Gill III, because of its added strength is often used in plastic and frozen sections.

D. **Bluing Reagent:** Ammonia solutions, tap water, Scott's solution, and lithium carbonate solutions.

1. **Use.** Bluing reagents change the reddish – purple hematoxylin to a blue or purple blue color. It is a pH dependent reaction and occurs in an alkaline solution.

a. **Troubleshooting.** Reddish color of a stained section is due to inadequate bluing. Bluing reagents should have a pH of approximately 8. It is not possible to over-blue a section. The bluing reagent can only blue the amount of hematoxylin in the tissue. If the section is too blue, there is too much hematoxylin in the section. Sections may fall off it left too long in a harsh bluing agent, such as an ammonia solution.

2. **Recommendations for bluing:** With dilute aqueous solutions (e.g. water, Scott's solution) no more than one minute is required. More potent reagents (ammonia solutions) often require less time. Sine the bluing reaction has a specific end point (i.e. it cannot be over done), the timing of the bluing step can easily be determined visually when the tissues turn "blue".

3. **Varieties:** There are numerous formulations of bluing reagents available. The active ingredient in any bluing reagent is the alkaline material – either ammonia or alkaline salts such as lithium or magnesium carbonate. The choice of whether to use tap water as a bluing reagent will depend upon the quality of the local tap water (See Water). Formulations of alcoholic bluing reagents work faster and are therefore better on linear stainers and for use by those individuals who use quick dip methods.

E. **Hematoxylin differentiator:** acetic acid, hydrochloric acid

1. **Use.** In the regressive hematoxylin staining method one purposely overstains the tissue sections, the removes the excess stain by using an acid rinse. An acid rinse is also used to remove non-specific hematoxylin staining (e.g. staining of the glass slide) with progressive or regressive stains.

2. **Troubleshooting:**

   a. Destaining will be excessive if:
      (1) Concentration of acid is too great
      (2) Concentration of water is too great as with alcohol-based differentiators
      (3) Destaining time is excessive
      (4) Decolorization agent is not completely removed with post water rinse

   b. Destaining will not be sufficient if:
      (1) Concentration of acid is sufficient
      (2) Concentration of alcohol is too great as with alcohol-based differentiators
      (3) Destaining time is inadequate
      (4) There is an excess of albumin/gelatin on the slide

3. **Recommendations for hematoxylin differentiator:** A water-based acid rinse acts quickly to destain the tissue section, making timing critical to the second. To allow more control and reproductively, alcohol-based acid solutions are used which slow down the rate of decolorizing. Therefore, most acid rinses are made up in 70% pr 95% alcohol. The concentration and type of acid (hydrochloric or acetic) used will determine the timing in the acid alcohol solutions. Acetic acid is weaker than hydrochloric acid; thus, its concentration must be higher. If purchasing commercially available hematoxylin, check manufacturer's directions for the recommended acid to use in the differentiation step.

4. **Varieties:** The type of acid rinse used will be determined partially by the method of staining. Hand staining, with the quickness of the hand, can handle the stronger hydrochloric acid and more aqueous-based solutions. In automatic staining it is necessary to use alcohol-based acid solutions, and/or weaker acids (e.g. acetic acid) or weaker concentrations of acids.

F. **Water:** Tap water, distilled/deionized water

1. **Use:** Water is used in H&E procedures in critical steps of rinsing before and after hematoxylin staining, as a possible bluing reagent, and in diluting alcohols.

2. **Troubleshooting**
   a. Tap water often contains elements that can destain hemztoxylin. Iron acts as mordant, sulfur tends to acidify water, and chlorine is a bleach.

   b. Tap water may not be alkaline enough (pH greater than or equal to 8) to act as a bluing reagent. The pH of tap water can fluctuate daily and seasonally.

   c. Water may contain particulate material that may adhere to the tissue sections.

3. **Recommendations for water:** Run water from the tap for a minute, then check its pH to determine if it is alkaline enough to be a bluing reagent. If iron or sulfur is a common element in the tap water, it is best to use deionized or distilled water for all steps after staining. Distilled/deionized water generally produces consistent results.

G. **Eosin:** Eosin, eosin-phloxine

1. **Use:** Eosin in the H&E procedure is referred to as a counterstain. It stains nearly everything that hematoxylin will not stain. When applied correctly, eosin produces three different hues which can be used to differentiate various tissue elements; red blood cells stain dark reddish orange, collagen stains a lighter pastel pink, and smooth muscle stains bright pink.

2. **Troubleshooting**
   a. Weak staining of eosin can be due to:
      (1) pH of the eosin – too high
      (2) Alcohol rinse after the eosin – too long or too aqueous
      (3) Contaminant in the alcohol rinse
      (4) Deteriorating eosin due to excessive carryover
      (5) Thin sections
      (6) Inadequate staining time

   b. Excessive staining of eosin can be due to:
      (1) Stronger dye solution due3 to excessive evaporation
      (2) Use of isopropyl alcohol as the rinsing agent
      (3) Thick sections
      (4) Excessive staining times
      (5) pH of the eosin – too low

   c. Undifferentiated eosin staining (one color) can be due to:
      (1) Poor fixation

(2) Overstaining due to:
(a) Aqueous eosin stain
(b) Excessive staining time
(c) Stain too strong

3. **Recommendations for eosin staining:** Generally, there are only three significant variables that influence proper eosin staining: eosin staining times, concentration of alcohols following the stain, and time in these alcohols. Eosin is very soluble in water. Therefore, the more water there is in the alcohol following the eosin, the more eosin there will be removed from the tissue. If isopropyl alcohol must be used due to cost or the unavailability of ethanol, staining protocol must consider the limitations of isopropyl alcohol as a rinsing alcohol. Anhydrous isopropyl alcohol has a poor solubility for eosin, and therefore does not rinse off the excess eosin. Diluted isopropyl alcohol (70%, 95%) can remove excess stain.

4. **Varieties:** There are numerous formulations of eosin available. Solutions can be either alcoholic or aqueous. Phloxine, picric acid, and/or orange G may be added to eosin solutions to give stronger red or orange tones.

II Rotation/Replacement of Solutions Guideline

A. **All staining solutions or reagents have a definite useful life.** Use the appearance of the symptoms under the sections **Troubleshooting to** assist your lab in determining rotation or replacement of solution schedules. Rotation/replacement schedules vary among labs because of work volume, type of solutions used, and mode of staining (automatic or manual).

B. **Wash and rinse thoroughly each staining vessel** at the time of rotation. Inadequate washing or rinsing can leave residues that can alter the chemical nature of new solutions placed in the vessel. Multiple water rinsing is mandatory when using detergents and acid rinses.

C. **Alcohol dehydration/clearant rotation.** A general rule is to rotate the final anhydrous alcohol when it acquires a pink cast from eosin carryover. Rotate the adjacent clearants at the same time. Whenever the appearance of solution looks unusual (e.g. milky alcohols, appearance of "beads" of water or clearant), discard and replace that solution immediately.

D. **Hematoxylin/Eosin Replacement.** Replacement schedules vary among labs because of work volume, type of solutions used, and mode of staining (automatic vs. manual). The information provided in the stain's **Troubleshooting** section along with microscopic examination of the control slide will help determine a regular replacement schedule for one's lab.

E. **Bluing Reagent.** If tap water is used, it should be changed with each rack/set of slides. Prepared bluing reagents should be changed at least daily.

III. Record Keeping

A. Daily activities.
1. Record on the daily staining log the following information. A staining log should be kept for each staining setup or automatic stainer.
a. the brand and lot number of each stain used

b. the pH of the eosin and hematoxylin
c. filtering of the hematoxylin
d. temperature and pH of water
e. the rotation/discarding/replacement of reagents
f. with multiple automatic stainers, record which slides are stained on which stainer

2. Run an H&E control slide and document results. (For thorough records, a control slide should be run in each slide rack.)

3. Provide the pathologist with a slide evaluation checklist to document the quality of the slides.

B. Control of purchased materials

1. Have written specifications for reagents.
   a. Denatured alcohol should include the denaturants and their percentages.

   b. Generic reagents (xylene, acids, bases, salts)
      (1) Chemical formulation with any waters of hydration
      (2) Grades (e.g. Reagent, Technical, USP, etc)
      (3) CAS number

   c. Dyes
      (1) C1 number
      (2) Certifications by the Biological Stain Commission

2. Mark reagents to indicate the date of receipt. High-light expiration date. Unless in conflict with expiration date, use on a first used basis. Date each reagent upon opening.

IV. General comments in preparing/using solutions.

A. Determining optimal staining times: The length of staining time will be dependent upon the desired intensity. Work closely with the pathologist or diagnostician to determine the preferred staining time. During trials to determine staining times, use all fresh solutions in the staining sequence. Use serial sections or control slides during trial staining. Recommended staining times can be obtained from textbook references or manufacturer's directions. When trying to correct any fault in the stained slide, make only one change at a time in the staining schedule. Record each change and the results before making subsequent changes.

B. Use all solutions at room temperature.

C. Have complete written specifications for all reagents prepared in the lab.

1. Stains: Record the dye content and adjust the concentration as warranted with new batches in dye.

2. Hematoxylin note: Hematoxylin is light sensitive and subject to oxidation. Stains should be stored in brown or opaque bottles. Oxidation may be slowed by keeping the stain in dishes, covered as much as possible, during staining and when not in use.

D. **Have quality control test for all reagents prepared in the lab.** It is an individual lab's decision on which tests, if any, to perform on commercially purchased reagents.

1. Alcohols (anhydrous and non-anhydrous)
   a. Odor
   b. Color
   c. Specific gravity. This will vary with the formulation of alcohol used and the temperature.

2. Stains
   a. pH
   b. Color
      Alum hematoxylin filter paper test: When dropped on a filter paper, it will produce a pattern by diffusion of a maroon color ending in a dark purple edge.
   c. Tissue test

E. **Labeling requirements**

1. For laboratory-prepared reagents
   a. Name of reagent
   b. Date bottle with preparation date
   c. Initials of the person preparing solution
   d. State expiration date
   e. If applicable, date bottle upon first use.

2. For commercially purchased reagents
   a. Name of reagent
   b. Highlight expiration date
   c. Date bottle received
   d. Date bottle opened/first used

F. In order to have **consistently stained sections** day after day, it is necessary to make microscopic checks after critical steps in the staining procedure (e.g. after bluing in a progressive program and after the hematoxylin differentiator in a regressive program). The presence of a functioning microscope is strongly advocated in the staining laboratory.

V. Suggested Reference Books

Bancroft JD, Stevens A: *Theory and Practice of Histological Techniques.* 4th Ed., Churchill-Livingstone, New York, 1995

Carson FL: *Histopathology A Self-Instructional Text,* 2nd Ed., ASCP Press, Chicago, IL 1990.

Luna LG: *Histopathologic Methods and Color Atlas of Special Stains and Tissue Artifacts.* American HistoLabs, Inc., Gaithersburg, MD, 1992

Prophet EB, Mills B, Sobin LH (eds): *Armed Forces Institute of Pathology Laboratory Methods in Histotechnology* .American Registry of Pathology, Washington, DC, 1992

Sheehan, Dezna and Hrapchak, B. *Theory and Practice of Histotechnology.* 2nd Ed., Battelle Press, Ohio, 1980.

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