SUBJECT: HEMATOXYLIN AND EOSIN STAINING

I. PRINCIPLE:
Since the H&E stain is performed routinely on the majority of tissue that goes through this lab, it is essential to have an excellent quality control program for staining. Adherence to this previously prescribed quality control measure is assurance to this desired end.

Hematoxylin:
Hematoxylin, a natural dye, which was first used about 1863, is without a doubt the most valuable staining reagent used in histologic work. It has little affinity for tissue when used alone, but in combination with aluminum, iron, chromium, copper, or tungsten salts, it is a powerful nuclear stain and chromatin stain. It has polychrome properties, which may be brought out with proper differentiation. The active coloring process, known as “ripening”, takes several days or weeks unless it is hastened by the addition of an oxidizing agent, such as mercuric oxide, hydrogen peroxide, potassium permanganate, sodium perborate, or sodium iodate.

The most common formulas for staining with Hematoxylin are combinations with aluminum in the form of alum.

The H & E is an effective stain for demonstrating the major histological structures, particularly nuclei which are most important structures in the viewing of histological sections for pathological changes. Since the H & E is almost always the initial stain used, it will invariably provide clues as to which other stains are required.

It is believed that the H&E is a "salt dye" mechanism of staining. Positively charged Hematoxylin will stain negatively charged tissue components, and negatively charged Eosin will stain positively charged tissue components.

There are two methods of staining when Hematoxylin is employed; they are progressive and regressive staining. Progressive staining is accomplished by employing a solution, which contains an excess of aluminum salts or acids, thus increasing the selectivity for nuclei. After staining with Hematoxylin, the slides are washed well in water and then counterstained.

Regressive staining on the other hand is accomplished by overstaining in a relatively neutral solution of Hematoxylin, then removing the stain from the other constituents with acid alcohol or other differentiating agent. The subsequent neutralization is accomplished by means of ammonia water, lithium carbonate, or buffered solution, and then the counterstain is applied.
**Eosin:**

Eosin, an acid synthetic dye, stains cytoplasm with different degrees of intensity. It stains readily and brilliantly after Zenker fixation. The reason for this is, that heavy metals such as mercuric salts combine with acid groups and therefore increase acidophilia. A longer time is required to stain formalin-fixed tissue.

Eosin is one of the most valuable counterstains known. Besides its widespread used as a counterstain for Hematoxylin or other basic (nuclear) dyes, it is used in a large number of blood stains, such as Giemsa, Jenner, and Wright stain.

Eosin is used in both aqueous and alcoholic solutions. It is usually employed in 0.5% and 1.0% solutions. However, when eosin (or Phloxine) is applied before an aniline dye such as methylene blue, a strong 2.5% to 5.0% solution must be used. Overstaining is necessary here, since the subsequent treatment in methylene blue extracts much of the acid stain.

Some of the eosin stain is removed from the tissue rather rapidly when it is dehydrated in 95% alcohol and more slowly in absolute alcohol. Therefore, you must allow for this by slightly overstaining the sections and rapidly dehydrating them in 95% alcohol and the absolute alcohol.

The choice of Hematoxylin and counterstain is a matter of personal preference. The Markey Biospecimen Histology Laboratory uses Harris' Hematoxylin.

### II. ROLE:

A. Research Analyst

### V. SPECIMENS (Samples):

10% Neutral Buffered Formalin fixed tissue, but may be used after any fixation. Cut paraffin sections at 3-6 microns and dry upright at 60°C for 20 minutes.

### VI. MATERIALS, REAGENTS, EQUIPMENT and SAFETY PRECAUTIONS:

A. Equipment and Materials
   1. Leica Autostainer XL for automated staining
   2. Coplin Jars for manual staining
   3. Slide holder racks
   4. Leica CV 5000 for automated coverslipping
   5. Coverlips

B. Reagents
   1. Cytoseal permanent mounting media
2. HEMATOXYLIN
Storage: At room temperature, in glass bottle.
See the Rapid Cold Method – Preparation of Harris’ Hematoxylin

3. 80% ALCOHOL
Storage: At room temperature, in glass bottle.
100% Alcohol……………………………………..800.0 ml
Distilled Water……………………………………200.0 ml

4. ALCOHOLIC EOSIN Y SOLUTION, STOCK
Storage: At room temperature, in glass bottle.
Eosin Y, water soluble………………………………10.0 g
Distilled Water……………………………………200.0 ml
95% Alcohol……………………………………..800.0 ml
80% Alcohol……………………………………3000.0 ml

Mix Eosin Y with distilled water, then add 95% Alcohol. Mix well. Add 80% Alcohol and mix well.

5. EOSIN Y SOLUTION, WORKING
Storage: Working Eosin solution is changed 3 times per week and is made fresh each time.
Eosin Stock solution………………………………..400.0 ml
Glacial Acetic Acid, concentrate……………………2.0 ml

6. 1% ACID ALCOHOL
Storage: At room temperature, in glass bottle.
80% Alcohol……………………………………99.0 ml
Hydrochloric Acid, concentrate…………………..1.0 ml

7. 1% AMMONIA WATER
Storage: Made fresh daily
Distilled Water……………………………………99.0 ml
Ammonium Hydroxide, concentrate………………1.0 ml

SAFETY PRECAUTIONS
Care must be used to avoid bodily contact with acids, xylene, alcohols and dyes as irritation to skin and eyes may occur. Wear proper protective equipment.
VII. QUALITY CONTROL

A. Tonsil and cervical biopsy, on one slide should be stained daily and analyzed microscopically to ensure proper preparation of reagents.
   - Nuclei: blue with some metachrosis
   - Cytoplasm: various shades of pink, identifying different tissue components

B. All slides are inspected for quality according to BCP H.15 Quality Control for Histologic Stains.

X. PROCEDURE

A. Staining
   1. Xylene, two changes to deparaffinize (must be fresh), three minutes each.
   2. Two changes of absolute alcohol, 15 seconds each.
   3. Two changes of 95% alcohol, 15 seconds each.
   4. One change of 80% alcohol, 15 seconds each.
   5. Rinse in running tap water for 15 seconds.
   6. Stain in Hematoxylin for 7 minutes.
   7. Rinse in running tap water for 20 seconds.
   8. Dip quickly in 1% acid alcohol.
   9. Rinse in running tap water for 30 seconds.
   10. Blue in 1% ammonia water, dip for 5 seconds.
   11. Rinse in running tap water for 2 minutes.
   12. Dip in 80% Alcohol for 15 seconds.
   13. Stain in Eosin Y working solution for 3 minutes.
   14. Dip in two changes of 95% alcohol, for 15 seconds each.
   15. Dip in 3 changes of absolute alcohol, for 30 seconds each.
   16. Clear in 2 changes of Xylene, 2 minutes each.
   17. Mount with synthetic resin.

B. Coverslipping
   1. Use Leica CV 5000 coverlipper for mounting of slides when possible
   2. For manual coverslipping
      a. Apply 1-2 drops off cytoseal to tissue, avoiding air bubbles.
      b. Place coverlip at edge of slide
      c. Allow the capillary rising action of the cover glass against the slide to cover the tissue.

PROCEDURAL NOTES:
The Leica Autostainer XL is employed for routine H&E staining. If manual staining is required, the given procedure can be followed by filling coplin jars with the indicated solutions and manually moving slides from jar to jar for the indicated times.
If sections become overstained with eosin and it cannot be readily removed in 95% alcohol, place sections in a diluted alkaline solution (0.1% ammonia water in 95% alcohol).
XIII. REFERENCES:


XV. HISTORY BLOCK

<table>
<thead>
<tr>
<th>Replaces:</th>
<th>NEW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Changes:</td>
<td></td>
</tr>
</tbody>
</table>

XVI. APPROVAL BLOCK:

<table>
<thead>
<tr>
<th>Written by: Dana Napier</th>
<th>Date: 10-12-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Revised by:</td>
<td>Date:</td>
</tr>
<tr>
<td>Approved by:</td>
<td>Effective Date:</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Medical Director</td>
<td></td>
</tr>
<tr>
<td>Approved by:</td>
<td>Date:</td>
</tr>
<tr>
<td>Supervisor</td>
<td></td>
</tr>
<tr>
<td>Annual Review</td>
<td></td>
</tr>
<tr>
<td>Medical Director:</td>
<td>Date:</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Date version removed from manual:</td>
<td></td>
</tr>
<tr>
<td>Date procedure retired:</td>
<td></td>
</tr>
</tbody>
</table>