James Hogg iCAPTURE Centre for Cardiovascular and Pulmonary Research

STANDARD OPERATING PROCEDURE

Title:	**Hematoxylin and Eosin Staining of Paraffin and Frozen Tissue Sections**		
Procedure:	BB_HIST.005.01	**Supercedes:**	none
Originator and Date:	Crystal Leung 29OCT2008	**Effective Date:**	29OCT2008
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Revision History		
Date	**Reviewer**	**Summary of revision**
21Apr2009	Crystal Leung	Reformatted to iCAPTURE format

Purpose

This standard operating procedure (SOP) establishes the standard for Hematoxylin and Eosin staining performed by Biobank personnel.

Responsibilities

This procedure is applicable to the following:

- Biobank personnel who are responsible for Hematoxylin and Eosin staining of tissue sections.

Safety

For safe handling of human biological material refer to “Handling Biohazardous Materials” SOP BB.001.01. Wear PPE (lab coat, gloves, goggles, etc.) when working with biohazardous materials and chemical substances. Refer to Material Safety Data Sheets for safe handling, disposal and storage of chemical substances.

Definitions

Alcohols	Used to introduce water into tissue sections (hydration) when a series of decreasing concentration is used and the removal of water from the tissue sections (dehydration) when a series of increasing concentrations is used.
Bluing Reagent	A base solution that changes the reddish-purple hematoxylin to a blue or purple-blue colour. It is a pH dependent reaction and occurs in an alkaline solution.
Clearing Agents	A solution miscible with the embedding medium (paraffin) and the dehydrant (alcohol). Used at two different stages in H&E staining a) Deparaffinization - removal of paraffin and b) Clearing - displacement of alcohol from the tissue sections with the clearing agent before coverslipping with mounting media. Most common clearing agent is xylene or citrosolve in deparaffinization.
Eosin	Is the counterstain for the H&E stain. It stains nearly everything that hematoxylin will not stain. Three shades of pink: red blood cells – dark reddish orange, collagen – lighter pastel pink, and smooth muscle – bright pink.
Hematoxylin	Stains the nucleus of the cell a shade of blue to purple-blue.
H&E	Hematoxylin and Eosin
Hematoxylin differentiator / Acid Alcohol	An acid solution. Used in regressive hematoxylin method where one purposely over stains the tissue sections and removes the excess stain by using an acid rinse. It is also used to remove non-specific hematoxylin staining (e.g. staining of the glass slide) with progressive or regressive stains.
MSDS	Materials Safety Data Sheets
PPE	Personal Protective Equipment
SOP	Standard Operating Procedure
Water	Running tap water, distilled/ deionized water

Materials and Equipments

The materials, equipment and forms listed in the following list are recommendations only and may be substituted by alternative/equivalent products.

Reagent containers	Surgipath Gill's III Hematoxylin
Staining racks	1% Eosin in 80% Isopropyl Alcohol *see Note 1
Filter paper for dye filtration	Acid Alcohol (1% HCl in 70% isopropyl alcohol) *see Note 2
Alcohol (Isopropanol)	Base (0.5 % lithium carbonate in 100mL distilled water) *see Note 3
Automated Coverslipper	Xylene/Citrosolve
Fluoromount Aqueous Mounting Media (Frozen sections)	PPE= Personal Protective Equipment (Lab coat/gown, gloves, goggles, closed toe shoes, etc.)
Glass coverslips	Tissue Tek Glas Mounting Media

Notes:

1) Eosin

1% Eosin in 80% Isopropanol
-see appendix 2 for % W/V calculation
-add 1 mL of glacial acetic acid to every 100 mLs of solution

2) Hematoxylin Differentiator (Acid Alcohol)

1% HCl in 70% isopropanol
-measure 1 mL of concentrated hydrochloric acid and add to 99 mL of 70% isoproanol

3) Bluing Reagent (Base)

0.5% lithium carbonate in 100mL distilled water
-pH of 8.0-10.0

Procedures

Notes:

i) Rotation / Replacement of Solutions: Follow established site rotation or replacement of staining reagents.

ii) Record Keeping (QC/ QA)
See appendix for sample H&E staining log sheet.

1. Cut paraffin sections at 4 µm thick or frozen sections at 5 µm thick and pick up on labeled slides. (Section thickness may vary depending on the type of biopsy and model of microtome. Section thickness of 4-5 µm is typically cut.)

2. Dry paraffin slides in 60°C oven for 30 minutes, then place slides in staining racks.
3. Place slides of frozen sections in staining racks and let air dry in room temperature overnight and then fix them appropriately if necessary – ie. In 10% neutral buffered formalin for 10 minutes.
4. H&E staining for frozen sections – see step 8.
5. Deparaffinize the paraffin sections as described below prior any staining procedures:

Station	Solution	Time
1-2	Citrosolve	10 min each station
3	100% Isopropanol	5 min
4	100% Isopropanol	1 min
5	90% Isopropanol	1 min
6	70% Isopropanol	1 min
7	Tap Water	1 min

6. Manual H&E staining for paraffin sections:

Step	Solution	Time
1	Tap Water	Wash/Rinse for 1 minute
2	Gill's Hematoxylin	5 minutes
3	Tap Water	Wash/Rinse for 1 minute
4	Differentiate in 1% Acid Alcohol	1-2 quick dips
5	Tap Water	Wash/Rinse for 30 seconds
6	Blue in Base	30 seconds
7	Tap Water	Wash/Rinse for 1 minute
8	Check slides microscopically for adequate nuclear staining. Nuclei should be blue to blue-black. Adjust staining by repeating steps 2 to 7 accordingly.	
9	Tap Water	Wash/Rinse for 1 minute
10	70% Isopropanol	30 seconds
11	1% Eosin in 80% Isopropanol	30 seconds
12	Drain away excess Eosin	
13	80% Isopropanol	10 seconds
14	90% Isopropanol	10 seconds
15	100% Isopropanol	10 seconds
16	100% Isopropanol	10 seconds

17	100% Isopropanol	10 seconds

7. Let air dry, then coverslip stained slides manually with glass coverslips or place stained slides in an automated coverslipper rack.

8. Manual staining for frozen sections:

Step	Solution	Time
1	Tap Water	Hydrate for 10 seconds
2	Gill's Hematoxylin	1 minute
3	Tap Water	Rinse for 10 seconds
4	Blue in Base	10 seconds
5	Tap Water	Rinse for 10 seconds
6	Check slides microscopically for adequate nuclear staining. Nuclei should be blue to blue-black. Adjust staining by repeating steps 2 to 7 accordingly.	
7	70% Isopropanol	30 seconds
8	1% Eosin in 80% Isopropanol	30 seconds
9	Drain away excess Eosin	
10	80% Isopropanol	10 seconds
11	90% Isopropanol	10 seconds
12	100% Isopropanol	10 seconds
13	100% Isopropanol	10 seconds
14	100% Isopropanol	10 seconds

9. Let air dry, then coverslip stained slides manually with glass coverslips and aqueous mounting media.
10. Staining Results:

Hematoxylin:
It stains the nucleus of the cell, specifically, the chromatin within the nucleus and the nuclear membrane. The neoplasm of the nucleus should remain unstained; this allows the staining pattern of the chromatin to be seen easily. The active ingredient in hematoxylin solutions is hematein complexed with a metal ion (eg. aluminum, iron, tungsten).
Aluminum is the most commonly used. Depending on the type of metal ion complexed with hematein, the shade of hematoxylin staining for:

a) Aluminum hematoxylins – blue
b) Iron hematoxylins – dark purple or blue-black

Eosin:
It stains nearly everything that hematoxylin will not stain and produces three different hues which can be used to differentiate various tissue elements;

a) Red blood cells stain dark reddish orange
b) Collagen stains a lighter pastel pink
c) Smooth muscle stains bright pink

Appendix I
H&E Quality Assurance

Date	Time in Oven / Stainer	Slide No.	Cut by	Time out of Oven / Stainer	Slide no. checked	Checked by

Appendix II
Percent Solution Calculations

Expressing Concentration
There are several ways to express concentration as a ratio of solute (solid or liquid) to solvent (in most cases "pure water"): weight to weight (W/W), weight to volume (W/V) and volume to volume (V/V).

Percent Solutions are expressed as parts per 100, "percent" or the symbol %.

Weight /Volume (%W/V) Solutions
Grams of solute per 100 ml of total volume

Example: Prepare 500 ml of a 5 % W/V Eosin aqueous solution.

For 100 ml of this solution 5 g of Eosin is required.
To prepare 500ml: weigh 5 x 5 = 25 g Eosin and make up to 500 ml with distilled water.

5g/100ml = X / 500ml

5g/ 100ml x 500 ml = X

25g = X

A one percent solution is defined as 1 gram of solute per 100 milliliters final volume. For example, 1 gram of sodium chloride, brought to a final volume of 100 ml with distilled water, is a 1% NaCl solution. One gram is the mass of one milliliter of water. Examples of 100% solutions are 1000 grams in 1000 milliliters or 1 gram in 1 milliliter.

Volume/Volume (%V/V) Solutions
Volume of a liquid solute per 100 ml total volume

Example: Prepare 200ml of a 5 % V/V acetic acid solution

For 100 ml of this solution 5 ml of concentrated glacial acetic acid are required.
To prepare 200 ml: 2 x 5 = 10 ml concentrated glacial acetic acid are measured and made up to a final volume of 200 ml with distilled water.

5 ml/ 100 ml = X / 200 ml
5 ml / 100 ml x 200 ml = X
10 ml = X

Weight/Weight (%W/W) Solutions
Grams of solute per 100 grams total weight

Example: Prepare 200 g of a 5 % W/W NaCl aqueous solution
5 % of the total 200 g must be NaCl.

This means:

5 /100 x 200 g = 10 g NaCl is needed to prepare the solution.

Therefore weigh out 10 g NaCl and dissolve in 190 g of distilled water for a total weight of 200 g,