

## Preparation of Materials for Staining:

### I. Wittman's Hematoxylin stock solution (Wittman 1965)

**Materials:** 4 g hematoxylin powder (Note: it is advisable to open the bottle for oxidation purposes prior to making the stock solution); 1 g iron alum,  $\text{FeNH}_4(\text{SO}_4)_2 \cdot 12 \text{H}_2\text{O}$ ; 100 ml 45% acetic acid.

**Procedure:**

- 1) pour hematoxylin powder and iron alum into a clean glass bottle;
- 2) add 45% acetic acid to dissolve the powder;
- 3) allow one to two weeks time for ripening prior to use.

### II. Hematoxylin working solution

**Materials:** 6 ml stock solution; 2 g chloral hydrate crystals.

**Procedure:**

- 1) add 6 ml stock solution to 10 ml centrifuge tube;
- 2) spin for 20-25 minutes at a top speed of 3000 rpm;
- 3) using a glass pipette, remove top 5 ml of working solution taking care not to agitate the 1 ml portion at the bottom which is usually loaded with unwanted precipitates;
- 4) place the top 5 ml portion into a clean test tube with screw cap;
- 5) discard the lower 1-ml portion properly;
- 6) dissolve 2 g chloral hydrate in 5 ml stock solution by shaking vigorously;
- 7) when all chloral hydrate crystals have been dissolved, stain is ready for use.

**Note:** For best results the working solution should be used within one week.

### III. Hoyer's Mounting medium (Stevens 1981)

**Materials:** 30 g gum arabic powder; 200 g chloral hydrate crystals; 16 ml glycerine; 50 ml distilled water.

**Procedure:**

- 1) dissolve Gum Arabic powder in distilled water;
- 2) add 20 g chloral hydrate crystals in order to control bacterial growth;
- 3) allow the solution to stand for 24 hours;
- 4) add 180 g chloral hydrate crystals and dissolve completely in solution;
- 5) allow 2-3 days storage time;
- 6) add glycerine and mix thoroughly;
- 7) to use Hoyer's mounting medium, dilute desired amount with distilled water (50 % Hoyer's solution, 50% distilled water).

**Note:** Used in conjunction with Wittmann's stain, Hoyer's medium enhances hematoxylin coloration in the Delesseriaceae after setting for 2-4 hours.

#### **IV. Piccolyte Mounting Medium** (as in Hommersand 1963)

**Materials:** Piccolyte solution 60 ml; xylene 40 ml

**Procedure:** Pour xylene into Piccolyte solution and mix thoroughly.

#### **V. Alcohol/Distilled-water series**

**Procedure:** 30%, 50%, 70%, 80%, 85%, 90%, 95% and 100% (absolute) alcohol prepared in individual dropping bottles.

#### **VI. Alcohol/Xylene series**

**Procedure:** 2:1 (Alcohol:Xylene), 1:2 (Alcohol:Xylene) and Xylene (100%) in individual dropping bottles.

#### **Staining Procedures:**

**Note:** specimens to be examined are placed in direct sunlight or under a tungsten lamp in 5-10 % Formalin-seawater until completely bleached prior to sectioning or staining.

#### **I. Staining with aniline blue** (Papenfuss 1937)

**Materials:** 100 ml bottle, 50 ml distilled water, aniline blue powder.

**Procedure:**

- 1) place 50 ml distilled water in the bottle and add the aniline blue into the bottle until the color turns deep blue;
- 2) better staining results when stain is applied directly to the sections or whole-mounted specimen before the coverslip is added;
- 3) after staining is complete, 20 % Karo or 100% glycerine is introduced from margins of the coverslip for permanent preservation, or the alcohol series is run through and the specimen mounted in Piccolyte to allow structures within multi-layered tissues to be observed.

## **II. Stain with Wittmann's hematoxylin solution** (adapted from Coomans 1986, Hommersand & Fredericq 1997a)

### **Procedure:**

- 1) after placing materials on slide, remove excess water with an absorbent paper strip placed along one edge of the coverslip; excess water must be removed before adding the staining solution as the stain reacts with water and forms black precipitates;
- 2) while adding the staining solution at one edge of coverslip, be sure to have an absorbent paper strip on the opposite side to facilitate diffusion of stain. Few drops of stain to fully cover the sections are sufficient;
- 3) allow materials to stain well. Staining for nuclei usually takes from 30 minutes to four hours or longer depending upon individual species;
- 4) after sufficient staining, remove excess stain using absorbent paper as in procedures 1) and 2) above;
- 5) apply drops of 45% acetic acid to one side of the coverslip for de-staining purposes. Absorbent paper on the opposite side will draw off the excess acid. It is better to watch the de-staining process under a dissecting scope so that if the material becomes overly de-stained, more stain can be added;

- 6) after sufficient de-staining, introduce the Hoyer's mounting medium from one edge of the coverslip until the material is covered. The mounting medium enhances the colour and keeps the stain inside the cells. The process takes about one to two hours (or longer) depending on individual species;
- 7) after completing the enhancing process, use 45% acetic acid to wash out the Hoyer's Mounting medium and run up through an ethyl alcohol series (30% first, followed by 50, 70, 80, 85, 90, 95 and 100 %) in a small watch-glass. If on a slide, introduce the alcohol from one side of the coverslip. From 100% ETOH, introduce a 2:1 mixture of alcohol and xylene, followed by a 1:2 mixture and finally pure xylene;
- 8) after running the dehydration series, dissect the sections on a slide in xylene and mount in Piccolyte. Piccolyte clears the tissues so that several layers of cells can be focussed through to see internal details.

## MOLECULAR STUDY

Red algae are well known to present major problems in yielding satisfactory DNA extraction products for successful gene amplification using the commercially widely available DNA extraction kits, such as the DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). For this reason, taxa that did not yield visible DNA upon total DNA extraction using the QIAGEN Minikit were submitted to a labor-intensive CTAB-Cesium Chloride DNA procedure perfected by Wilson Freshwater (e.g. Freshwater *et al.* 1994, Hommersand *et al.* 1994, Fredericq *et al.* 1996, 1999). The adding of Proteinase K when grinding the samples using the Qiagen Minikit often improved the PCR yield (Hughey & Hommersand 1999).

**Total DNA Extraction Protocol** (adapted from Wilson Freshwater, pers. comm.)

### I. CTAB-Cesium Chloride DNA extraction procedures: