**LEAF CLEARING PROTOCOL**

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**MATERIALS**

* Microscope slides
* Petri Dish
* Bleach 6.5%
* Water
* Sodium hydroxide, pellets
* Safranin-O (powder)
* Ethanol 100%
* Tweezers

**SAMPLE** **PREPARATION**

Label both sides of the Petri dish with the sample code using an ethanol-resistant marker. Place leaves of the same species in the same Petri dish.

**CHEMICAL PROCESSING**

**Solutions**

1. Sodium hydroxide 2.5%: 25 g of sodium hydroxide (NaOH) in 1 L of water.
2. Bleach 3.25%: mix 500 mL of 6.5% sodium hypochlorite (NaClO) and 500 mL water (tap or deionized).
3. Ethanol/water 50%: mix 500 mL of water and 500 mL of 100% ethanol.
4. Ethanol/water 70%: mix 300 mL of water and 700 mL of 100% ethanol.
5. Ethanol/water 95%: mix 50 mL of water and 950 mL of 100% ethanol.
6. Safranin 0.1%: 1 g of safranin in 1 L of 100% ethanol.
7. Ethanol 100%: 1 L of 100% ethanol.

The proportions don’t need to be exact, so it is acceptable to measure liquids in a beaker and to round up or down (within reason) when weighing solids. Pour each solution (except for water, which should be renewed every time) into a different flask of the appropriate size, cover it with a lid, and label the flask with the name, concentration, and date of preparation of the solution.

**Protocol**

**I. Clearing**

1. Pour 2.5% NaOH into the petri dish until the leaves are fully immersed. Keep it at 37°C for 24 hrs or until the leaves become transparent. It is crucial to ensure that the leaves remain submerged to prevent them from drying out. For tougher leaves, over 24 hours may be necessary.
2. Rinse the leaf with tap water, but do not allow the water stream to strike the leaf. Let the leaf remain in the water bath at room temperature for at least 10 minutes.
3. Bleach the leaf in 3.25% sodium hypochlorite for 20 seconds to 10 min or until the leaf turns white. Once the leaf turns white, rinse it twice with tap water, making sure not to let the water stream strike the leaf. Keep the leaf in the water bath for another 10 minutes.

**II. Staining**

1. Remove the water from the petri dish and dehydrate the leaf in a graded ethanol series (50%, 70%, and 95%) for 30 minutes each.
2. Stain the leaf with safranin in 95% ethanol for a duration of 3 to 12 hours.
3. Rinse the leaf in 95% ethanol for a period of 10 minutes.
4. Destain in 95% ethanol with 3-6 drops of 37% hydrochloric acid for 10-15 seconds, or until the mesophyll destains to a clear white. This may vary based on the thickness of the leaf.
5. Immerse the leaf in 100% ethanol for two changes, ensuring it remains submerged for at least 10 minutes each time.
6. The leaf can be stored in 100% ethanol. Make certain the leaf is fully immersed in ethanol, and cover the petri dish with parafilm to prevent dehydration.