Protocol P-13-49

Preparation of Diatom Slides Using Naphrax™ Mounting Medium

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1. PURPOSE

1.1. Accurate identification and enumeration of diatoms requires mounting of cleaned material between a microscope slide and cover slip in a medium with a refractive index near that of glass, so that the features of diatom frustules or valves are clearly visible at high magnification. Naphrax™, a commercially-available toluene-based mounting medium with high refractive index, is currently used at the ANSP. This protocol details the steps necessary to produce high-quality diatom mounts from cleaned diatom material. This technique produces ‘permanent’ mounts, preserving the diatom specimens over many decades, at least.

2. SCOPE

2.1. Procedures described in this protocol include the dilution and dispersion of cleaned material onto glass cover slips, the mounting of cover slips onto glass microscope slides using Naphrax™ mounting medium, and the labeling of permanent mounts suitable for inclusion in the ANSP Diatom Herbarium.

2.2. This procedure applies to personnel involved with the preparation of diatom slides.

3. REFERENCES


4. SAFETY PRECAUTIONS

4.1. Personnel should be familiar with the information given in Reference 3.4.

4.2. Naphrax™ should be considered a hazardous substance because it contains toluene, an organic solvent. Toluene volatilizes readily when heated. For this reason, heating of Naphrax™ should only be performed under a positive-draw fume hood. Personnel should wear safety glasses and protective hand wear when working with liquid Naphrax™ at room
temperature, when heating Naphrax™ in a hood, or when in contact with solidified Naphrax™ toward the final stages of slide preparation.

4.3. Hot plate temperatures required for this procedure are high enough to cause severe burning of exposed skin. Use extreme care when manipulating slides on the hot plate and when working close to the hot plate.

5. **APPARATUS/EQUIPMENT**

5.1. Corning ceramic-top hot plate with temperature control.

5.2. Positive-draw chemical hood.

5.3. Aluminum drying plate (25.5 x 20.0 x 0.5 cm, solid aluminum; lines forming 48 squares, each 3.2 cm on a side, are etched on the surface. Each square is etched with an identifying number).

5.4. Glass microscope slides (1 x 3 inches; 2.5 x 7.5 cm)

5.5. Glass cover slips (18 mm x 18 mm) - No. 1 thickness, stored in covered glass jar filled with 100% ethanol.

5.6. Naphrax™ mounting medium.

5.7. Diamond scribe.

5.8. Disposable plastic pipettes.

5.9. Adjustable pipettor (0 - 250 µl); adjustable pipettor (200 - 1000 µl).

5.10. Pipette tips for adjustable pipettors.

5.11. Round-style tooth picks.

5.12. Forceps.

5.13. Polished, rounded wooden splints.

5.14. Wash bottle filled with distilled (DW) or reverse osmosis (RO) water

5.15. Single-edged razor blades.

5.16. Ethanol, 70%.

5.17. Acetone.

5.18. Kimwipe® tissues.

5.19. ANSP slide labels.

5.20. Wax (the kind commonly used for candle making and canning foods).

6. **METHODS**

6.1. Estimate amount of cleaned diatom material to deposit on coverslip.

6.1.1. Starting with cleaned material contained within 20-ml glass vials, estimate the volume of suspended material that will need to be deposited (“dripped”) on a cover slip to produce a slide of the appropriate cell density. The ideal density to be achieved on the
final mount is somewhat subjective and is based on the amount of debris in the sample, the preferences of the slide analyst and the way in which the slide is to be used (e.g., counting, documentation). Generally, between 5 and 10 diatom specimens should be present in a single high power microscope field (1000X). To make the estimate, shake the cleaned material to ensure a homogeneous dispersion of cells within the 20-ml vial. Immediately open the vial and withdraw either a 25- or 50-µl sub-sample using the 0- to 250 µl adjustable pipettor. Place the subsample on a slide and cover it with an 18 x 18 mm cover slip. Then observe this preparation under a compound microscope at 50X magnification. Look at a number of fields and observe the density of cells. Then calculate the amount of material that would need to be dripped so that the density of cells seen at this magnification would be approximately 30 to 40 per field. This estimate is referred to as the “drip count” (the amount of cleaned material to be placed on a cover slip). Accuracy of estimates improves with experience. In many cases, analysts will request that both a “heavy” slide (~40 cells/field) and a “light” slide (~30 cells/field) be made. Record the “drip count” estimates on the “Diatom Lab - Slide Preparation Notes” form (Figure 1). When slidemaking is complete, record the estimates and final amounts dripped on the “Diatom Slide Preparation Form” (See Figure 2, Protocol P-13-48). Also note observations of interfering materials (sand, silt, etc.) on this form.

6.1.2. In some cases, the number of diatoms in a sample is very sparse. This is usually because diatoms were rare in the habitats sampled, or the sample bottles contain a small amount of material. In these cases, additional procedures are required to either make a satisfactory slide for analysis or to determine that analysis of a sample is not practical. Follow these procedures if more than about 900 µl (this is the maximum amount that a coverslip can “hold” beneath it) would need to be dripped onto a coverslip to meet the above criteria.

6.1.2.1. If a satisfactory slide could be made by increasing the concentration of cleaned diatom material by two to five times, then do this by using a micropipettor to remove the required amount water from the vial of material after it has been allowed to settle for at least eight hours. Record the concentration factor on the “Diatom Slide Preparation Form.”

6.1.2.2. If a concentration of cleaned material greater than two to five times is required, then re-subsample the original sample (Protocol No. P-13-48). Take a subsample of a size sufficient to prepare satisfactory slides. Use all of the remaining sample only if absolutely necessary. Digest the subsample and prepare a new vial of cleaned material (Protocol P-13-42). Repeat procedure 6.1.1, above. If the concentration of cleaned material is still not sufficient, concentrate it, as described above. If still too dilute, combine the two vials of cleaned subsample materials. Record steps and volumes, and final concentration factor, on the “Diatom Slide Preparation Form.”

6.1.2.3. If, after following the steps above to concentrate the cleaned material, the density of diatoms on a cover slip still does not meet the criteria of 30 to 40 cells per field at 400 - 450X magnification, then proceed to make the densest slide possible and take it to a diatom analyst to evaluate (the Phycology Section Diatom Taxonomy Coordinator at the ANSP). The analyst will make a determination of whether it is practical to analyze the sample. They will quickly scan the slide in its entirety under 100X magnification, and estimate the total number of
individuals on the slide. Then they will make their determination of whether the slide is countable, taking into account the density of diatoms, evidence of dissolution, and amount of debris (silt, clay, broken remains of diatoms and other siliceous organisms) that would make it difficult to identify specimens accurately. As a general guideline, if accurate identifications are possible, and at least 100 specimens could be counted within four hours, they should determine that the slide be analyzed; otherwise it should not. If the diatom analyst determines that the slide should not be counted, inform the Phycology Section Project Manager immediately. They will call the NAWQA study unit biologist that submitted the samples to inform them of the problem. Only under very special circumstances will an analyst be asked to take the extraordinary measure of counting a slide for a very long time (more than four hours). Record results of the diatom analyst’s determination and rationale on the “Diatom Slide Preparation Form.”

6.1.2.4. When doing their evaluation of a slide with few diatoms, as described in the step above, a diatom analyst may occasionally see evidence suggesting that a sample contains lightly silicified diatoms that may not have survived the digestion process. In these rare instances, they may suggest that a “burn mount” be made to determine whether diatoms did exist in the original sample. (This is one reason why a small portion of the initial sample should always be saved, even for phytoplankton.) The burn mount procedure was used extensively to create slides for diatom analysis before the introduction of methods incorporating acids for the digestion of organic material. Even though this method does not rid sample material entirely of organic debris, diatoms on the slide can at least be identified as diatoms. For this method, follow the EPA (1973) procedure. Briefly, a known portion of the untreated sample is dripped onto a coverslip and allowed to dry at room temperature. When the sample is dry, it is placed onto a hot plate and left for about 30 min at ca. 570°C. The coverslip is mounted according to procedure 6.3 below.

After it is prepared, have the burn mount slide examined by a diatom analyst. They will determine if diatoms are present and whether analysis of the slide is warranted. Slides prepared using the burn mount method can not be counted if too much organic material remains on the slide. This is because it is not possible to make accurate taxonomic identifications. Generally, burn mounts are used only as a last resort, and to confirm that weakly silicified diatoms are not present in the sample. Record information on all burn mount attempts, successful or unsuccessful, on the “Diatom Slide Preparation Form.” Include at least date, name of preparer, volume of subsample used, and whether diatoms were observed.

6.2. **Deposit cleaned material on coverslip.** Use forceps to remove single 18 x 18-mm cover slips from the ethanol storage container, and carefully clean each by wiping with a Kimwipe®. Place each cover slip on a marked space of the aluminum drying plate. Be sure the aluminum drying plate is clean and dry to avoid cross-contamination. If the intended drip count is less than 600 µl, drip a small amount of distilled water onto the cover slip with a disposable pipette, sufficient to form a thin layer of water over the entire cover slip. Agitate the sample vial to a uniform dispersion and use the adjustable pipettor to quickly withdraw the required amount from near the central portion of the sample. Eject
this material smoothly and carefully onto the layer of distilled water already on the slip. By alternately drawing material up into the pipette and ejecting it, a homogeneous suspension is achieved on the cover slip. In the case where more than ~600 µl of original sample is required, the addition of distilled water is not necessary, and the sample can be ejected and mixed directly on the cover slip. In both cases, take care to ensure that the suspension covers the entire surface of the cover slip, including the extreme edges of the corners. Should the cover slip overflow, discard the cover slip, and repeat the procedure with a freshly cleaned cover slip. Discard the pipette tip when finished with each sample.

Once the aluminum drying plate is loaded with cover slip preparations, the plate should remain undisturbed until the cover slips are dry. At this point, drying of the slips can proceed at room temperature (a period of several hours will be required), or gentle heat (warm to the touch only) may be applied to hasten evaporation (a crook-neck lamp with incandescent light bulb placed 15 - 30 cm over the drying plate is one option). Once completely dry, put the aluminum plate with cover slips on the hot plate that has been preheated to 250 to 300ºF. Leave for 3 to 5 minutes. This procedure ensures that nearly all water is driven from the material on the cover slips and helps assure that the diatom frustules will adhere to the surface of the glass. Remove the aluminum plate from the hotplate and inspect the cover slips. If the pattern of diatoms distributed on any of the cover slips is not even and smooth, they should be re-dripped. If cover slip distributions seem unsatisfactory after repeated attempts, consult an algal analyst.

6.3. Mount coverslip on microscope slide.

6.3.1. Using a diamond scribe, etch microscope slides with Sample ID, Subsample ID and Slide Replicate ID (e.g., GS029231 DT1 a).

6.3.2. Mount coverslip on slide.

THE FOLLOWING STEPS MUST BE PERFORMED IN A POSITIVE-DRAW FUME HOOD!

Using a rounded wooden splint or disposable pipette, transfer a small amount of Naphrax™ (volume equivalent to ~2 to 4 drops of water) to the central portion of the etched side of the microscope slide. Using a rounded wooden toothpick, distribute the Naphrax™ over an area approximately equivalent to the size of the cover slip. Then remove the appropriate cover slip from the aluminum plate with forceps, being careful to handle the cover slip only at the extreme corners. Invert the slip and place it gently on the Naphrax™ covered portion of the slide. Then place the slide (cover slip up) on the hotplate and apply gentle heat until the evolution of bubbles resulting from the evaporation of the toluene solvent first occurs, and then significantly diminishes. Remove the slide from the hot plate, and, using the rounded toothpicks, gently position the cover slip and press it to form a uniform, thin layer of Naphrax™ beneath the entire cover slip. Make sure that the edges of the cover slip are brought parallel to the edges of the microscope slide. Care must be taken at this stage not to press so hard as to damage or dislodge the diatoms or cause warping of the cover slip. As this procedure is taking place, the Naphrax™ is “setting up” (becoming hard), and the ability to move the cover slip will diminish rapidly. At this point, set aside the mount to finish cooling.

6.3.3. Use a single-edge razor blade to carefully trim any excess Naphrax™ which has been squeezed out from beneath the cover slip. Great care must be taken to avoid “lifting”
the cover slip by inadvertently allowing the edge of the blade to move between the cover slip and the microscope slide. Once most of the excess Naphrax™ has been removed and discarded, and while still working under the hood, place the mount in successive baths of acetone, and then ethanol for no more than 10 or 15 seconds each. Finally, wipe the mount clean with a Kimwipe® tissue.

6.4. **Add paper label to slides.** Either before or after slides have been analyzed, depending on project requirements, prepare paper labels and attach them to the mounts following the exact specifications and examples contained in Appendix 1. The standard labels produced by the Phycology Section include those for diatometer projects, surveys (hand collections), general projects (miscellaneous types), and the USGS NAWQA program. Contractors often submit slides with only etched labels; paper labels are added by Phycology Section staff.

6.5. **Enter data from the “Diatom Slide Preparation Form”**. Enter data directly into the following fields of the “Slide Information” table in the PHYCLGY database: Sample ID, Diatom Subsample ID, Slide Replicate ID, Vol Cleaned Material, D/C Factor, μL dripped, Settled By (Worker ID), Mounted By (Worker ID), and Date Diatom Slide Completed.

6.6. **Assemble forms and transmit slides.** Put slides in plastic slide boxes; label each with name of project and subproject, Subproject ID, Box _ of _, date (month/year) box prepared, and name or initials of preparer. Sign and date the “Diatom Slide Preparation Form” and the “Diatom Lab - Slide Preparation Notes” form and put them in the “Diatom Analysis” folder. Print a “Diatom Slide Analysis Form” for use by the diatom analyst and add it to the “Diatom Analysis” folder also. Create this form using the “Diatom Analysis Form(...)” report in the PHYCLGY database. When slides are completed, transmit them and associated forms to the Phycology Section Project Manager, or inform them that slides are prepared.

6.7. **Preserve and store cleaned material.** After slides are analyzed according to the appropriate protocol, and no additional slides need to be made, process the vials containing the remaining acid-cleaned material for long-term storage. Working under a fume hood, add two - four drops of 100% buffered formalin to each vial (some contractors use alcohol as a preservative instead). Tightly cap the vials and seal them by immersing the top 1/3 of the vial in melted wax. Transfer the vials to the appropriate storage cabinet in the Phycology Section for long-term storage. Be sure that the cabinet and shelves on which they are stored are properly labeled with the study unit year and Subproject ID. See the Phycology Section Project Manager for assistance.

7. **QUALITY ASSURANCE/QUALITY CONTROL**

7.1. This procedure was developed in the laboratories of the ANSP and has been used for the preparation of several thousand slides. Naphrax™ is produced under quality control conditions specifically for the purpose of high resolution slides (Northern Biological Supplies of Islip Great Britain). Naphrax™ mounts have proven to be stable over long periods (there are 25 plus year mounts in the ANSP Diatom Herbarium) and has been the mounting medium of choice of European investigations for over 40 years. Before it’s production was halted in 1993, Hyrax™ was the most widely used commercially-available mounting medium and was used at the ANSP for many years before the switch to Naphrax™.
7.2. It should be understood that, given the microscopic size and large numbers of diatoms which are transferred from the cleaned material vials to the finished mount, there are a number of steps where contamination of the samples is possible. Laboratory rooms where raw or processed samples are handled should be kept as clean as possible. Laboratory bench surfaces should be kept clean and free of debris at all times. Techniques similar to those used for sterile experiments (bacteriological plating, etc.) should be followed to minimize the risk of cross-contamination of samples. All equipment coming into contact with sample material should be rinsed in DW or RO water at least three times. Disposable pipettes should be used when possible.

7.3. The distribution of specimens on the final mounted cover slips should represent the samples contained within the cleaned material vials. The degree to which this is true depends on how well the cleaned material is dispersed prior to sub-sample withdrawal, and how evenly the withdrawn material is dispersed on the cover slip. Great care should be taken to ensure that these two steps are completed properly.

7.4. For certain critical applications, the project protocol may call for duplicate slide sets to test for variation in quantitative data introduced by this procedure.
## Diatom Lab - Slide Preparation Notes

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**Notes:**

**Dripped By:**

**Date Dripped:**

**Project Number & Name:**

**Figure 1.** Diatom Lab - Slide Preparation Notes form.
APPENDIX 1

SLIDE LABEL FORMAT (ANSP DIATOM HERBARIUM)

Listed below are formats for slides labels for diatometer projects, surveys and special projects. Labels are 1 inch (2.54 cm) square and can accommodate 10 lines with 15 characters or spaces per line. Abbreviations should be used when necessary and clear.

I. Diatometer Projects

Line #1: State abbreviation, county name (or abbreviation)
   e.g. SC, Allend. Co.

Line #2: Waterbody
   e.g. Savannah River

Line #3: Station and substation code (diatometer code)
   e.g. Sta: 1RC

Line #4: Installation date (exposure date)
   e.g. exp: VIII-12-87

Line #5: Removal date
   e.g. rem: VIII-26-87

Line #6: (nothing; assumes no particular collector)

Line #7: Project name
   e.g. Sav Diatom. #35

Line #8: sides scrapped, microliters dripped
   e.g. 6 sides 1500 µl

Line #9: (nothing; reserved)

Line #10 preprinted Acad Nat Sci Philadelphia or ANS Phila.

Example:

SC, Allend. Co.
Savannah River
Sta: 1RC
exp: VIII-12-87
rem: VIII-26-87
Sav. Diatom. #35
6 slides 1500 µl
ANS Phila.
II. Surveys (Hand Collections)

Line #1: State abbreviation, county name (or abbreviation)
e.g. TX, Victoria Co.

Line #2: Waterbody
e.g. Guadalupe River

Line #3: Station/substation/collection #
e.g. Sta 1L Coll: 19

Line #4 and
Line #5: Microhabitat
e.g. on rocks, gravel and sand

Line #6: Collection date
e.g. XII-10-1987

Line #7: Collector
e.g. Coll: RR Grant

Line #8: Survey name and number
e.g. Sav Cur Sur #96

Line #9: Microliters dripped
e.g. 1500 µl

Line #10: preprinted Acad Nat Sci Philadelphia or ANS Phila.

Example: TX, Victoria Co.
Guadalupe River
Sta 1 Coll: 19
on rocks, gravel and sand
XII-10-1987
Coll: RR Grant
Sav Cur Sur #96
1500 µl
ANS Phila.
III. General Projects (Miscellaneous)

Line #1: State abbreviation, county name (or abbreviation)
   e.g. PA, Wayne
        OH, Clermont

Line #2: Waterbody or installation
   e.g. Swago Pond
        P&G Art Streams

Line #3: Station and substation or treatment
   e.g. Treatment: NPC1
         Stream 1, Rep 1

Line #4: Microhabitat (if necessary)
   e.g. nutrient pots
         glass slides

Line #5: Installation or collection dates (if applicable; exp = exposure date)
   e.g. exp: VIII-11-87
        VIII-31-87

Line #6: Removal date (if applicable)
   e.g. rem: VIII-31-87

Line #7: Collector (if applicable)
   e.g. JW Sherman

Line #8: Project name
   e.g. POCONOS 1987-88
        1988 P&G
        ART. STREAMS

Line #9: Sides scrapped, microliters dripped (if applicable)
   e.g. 6 slides 1500 µl

Line #10 preprinted Acad Nat Sci Philadelphia or ANS Phila.

Examples:
   PA, Wayne        OH, Clermont
   Swago Pond      P&G Art Streams
   Treatment: NPC1 Stream 1 Rep 1
   nutrient pots   glass slides
   exp: VIII-11-87 VIII-31-87
   rem: VIII-31-87
   Coll JW Sherman  1988 P&G
   POCONOS 1987-88 Art Streams
   6 slides 1500 µl
   ANS Phila.      ANS Phila.
IV. USGS NAWQA Program

Listed below is the format for slides labels for USGS NAWQA Program. Two labels are generated for each slide. One to be placed to the left of the coverslip and the other to be placed to the right of the coverslip. Abbreviations should be used when necessary and clear.

Left Label:

Line #1: State abbreviation, county name (or abbreviation)
  e.g. WI, Milwaukee

Line #2: Waterbody
  e.g. Lincoln Cr.

Line #3: Site Location ID
  e.g. GS40869415

Line #4: Microhabitat (if necessary)
  e.g. nutrient pots

Line #5: Collection dates
  e.g. 5/15/95

Line #6: Collector (if applicable)
  e.g. B. Scudder

Line #7: USGS NAWQA

Line #8: USGS NAWQA Sample ID
  e.g. WMIC0595ARE0001B

Line #9: ANSP Slide ID
  e.g. GS004503-DT1-b

Example:
  WI, Milwaukee
  Lincoln Cr.
  GS40869415
  5/15/95
  B. Scudder
  USGS NAWQA
  WMIC0595ARE0001B
  GS004503-DT1-b

Right Label:

Line #1: ANSP Diatom Herbarium Accession Number
  e.g. 100001b
Lines # 2 through #7: Reserved for names of taxa found on the slide (if applicable)

Line #8: Determiner (If applicable)

Line #9: ANSP