LacCore SOP Collection

Pollen Preparation Procedure

Purpose

The purpose of the procedure is to make pollen analysis (microscopic counting of pollen grains) as easy as possible. Sediment residue with a high pollen concentration is generated by using a series of chemical and mechanical treatments to selectively remove as much of the non-pollen components of sediment as possible. This works because the pollen grains are: (1) highly resistant to chemical breakdown and thus are preserved when other sediment components are destroyed; (2) are denser than water, so can be centrifuged easily; and (3) range between 8 and 140 µm in size so can be effectively sieved. Because sediment varies a great deal, even in one core, the procedure must be customized to the sediment. Success makes counting pollen simpler, more accurate, and less time consuming. Study of the changing frequencies of different pollen types throughout a sediment core allows for the study of past vegetation and climate changes.

Safety

The following section regards sound laboratory techniques, safety practices, and manners. You are responsible for following these procedures. The chemicals, glassware, and equipment are potentially hazardous. Lab staff must specifically train you before beginning the procedure.

Required personal protective gear: lab coat, chemical splash goggles, and gloves must be worn at all times. You must wear closed toe shoes and long pants. If you have long hair, make sure to tie it back.
A chemically resistant vinyl apron with full-length sleeves, face shield (in addition to goggles), and rubber gloves over nitrile gloves must be worn when using hydrofluoric acid. If you are found without any of these required personal safety devices you will relieved of duties.

HF is poisonous. It has extreme health hazards and requires special training. It penetrates skin easily and has an affinity for calcium. It will attack bone and can form deep sores that ulcerate and take months to heal. Keep several versions of SDSs (material safety data sheet) available as the exposure treatment suggestions vary somewhat. Read and understand them. Read warnings on the bottle. Any exposure merits an emergency room visit (take the SDS, so they know what to do). Basic procedure involves extensive rinsing of exposed areas with COLD WATER, then soaking in iced magnesium sulfate (Epsom salts) solution for an hour or more (cold slows the reaction). Keep the following three items on hand (1) Calcium gluconate in Surgilube (mixed by a pharmacy) that can be put on hard-to-reach areas such as under fingernails, (2) a jar with soda ash to pour on small spills in the fume hood - soaks up and neutralizes, and (3) HF Acid Eater Neutralizer to use for larger spills. If you have an exposure, flush the area immediately and continuously with cold water until you are ready to go to the Emergency Room. If you have a spill outside of the fume hood, notify the people around you and leave the room; do not attempt to neutralize with soda ash. Then call the Department of Environmental Health and Safety.

Acetic Anhydride can explode in the presence of water.

All reactions must be done within a fume hood. Many of these chemicals are not compatible with each other. Acetic anhydride can explode on contact with water. Follow the steps in order to prevent mixing incompatible chemicals. Read and understand all SDS.

Always be sure to turn off the hot plate. Empty the water bath pan and leave it up side down to drain on the counter (not on the hot plate).

Record Keeping

Use a lab notebook to log each batch of pollen samples and make sure to record each step of the procedure as it is done. Keep accurate logs with each set of samples. Include dates, any errors, and anything you note of importance about the samples. Write down what you do at the time you do it**.** When finished, enter the information into the FileMaker Pro Pollen Processing database. Each log must have the following information for each sample: site name and location, core ID, sample depth, sample volume,

spike amount and batch, and any other information that is specific to those samples. Lab notebooks become a permanent record of the lab and may need to be referenced after you leave.

All labeling is important. A perfectly prepared sample that is mislabeled is useless. Sample vial labels are permanent and part of the research archives. The label should include site name, location, core #, sample depth, sample volume, preparation date. Make sure to double check that the label matches what you have written in your lab notebook. Additionally, during processing, it is critically important to label all tubes and beakers correctly to minimize errors related to the mixing up of samples.

There is no substitute for careful personal instruction in the subtleties of this procedure. Samples vary more than you might expect, and it is often necessary to give some of them individualized treatments. While learning, we encourage you to ask if you are unsure what to do. We want you to know that mistakes happen, even to those who have years of laboratory experience. The critical requirement is the *mistakes be noted and discussed* when they happen so corrections or adjustments can be made. It is generally best to start over.

Quantitative Procedure Notes

This is a quantitative procedure**.** This means that in theory, there is no loss of pollen during the course of the treatment. Be aware of the causes of lost sample material as discussed below. Concentrate on what you are doing.

Pollen data is only as good as the preparation. An analyst must be confident that the differences between samples are due to natural differences in the samples and not due to differences in the pollen preparation procedures. The same lab technician should process all the samples in a project.

Contamination problems come from poor housekeeping. The lab and glassware must be kept clean. Carefully follow the cleaning procedures.

Reagents

Reagents are dispensed from repipettes, squeeze bottles, or special anti-drip bottles if dangerous. Do not touch the tip of the squeeze bottle to the side of a test tube; this prevents contamination.

Reagents used as supplied by the manufacturer include 48% hydrofluoric acid (HF), 98% sulfuric acid, glacial acetic acid, TBA - tertiary butyl alcohol, acetic anhydride, 100% ethanol, 95% ethanol, 2000cs silicone oil, and soda ash (sodium carbonate; waste reaction technical grade – 100 lb. bags).

Some of the reagents need mixing (weight-to-volume or volume-to-volume). Mix the chemical ***as supplied*** with high purity distilled water to obtain desired percentage. Note that the result is not necessarily a true percentage of the original. We make 2-3 liters ahead and use over several months. Do all mixing in the hood. Wear gloves, lab coat and chemical splash goggles.

1. 10% KOH = 100 g potassium hydroxide pellets plus 900 mL DI water. Make sure the accuracy is to the nearest gram and measure DI water in a graduated cylinder. Put weighed pellets in container and SLOWLY add DI water. Carefully swirl until pellets dissolve.
2. 10% HCl = 900 mL DI water plus 100 mL conc. 38% hydrochloric acid. Measure chemicals using a graduated cylinder. Pour acid slowly into DI water.
3. 10% sodium pyrophosphate = 900 mL DI water plus 100 g sodium pyrophosphate. Make sure the accuracy is to the nearest gram and measure water in a graduated cylinder. (This reagent is optional)
4. Acetolysis mixture: REACTS EXPLOSIVELY WITH WATER. Mix in a DRY 100 mL graduated cylinder. The ratio is 9:1 of concentrated acetic anhydride: 98% sulfuric acid. Mix just prior to use. Directions are in the text.

Equipment

Use a centrifuge with swinging heads (fixed heads do not work for pollen processing). Temperature control is not needed and speeds are relatively low. LacCore uses 3500 rpm (~2450 rcf) setting to centrifuge pollen samples. We currently use an Eppendorf 5810 centrifuge, after much trial and error. Please see the Centrifuge SOP for proper use, care, and maintenance. You are responsible for following the information in the Centrifuge SOP. It is essential that the centrifuge buckets be balanced by weight.

You need centrifuge vapor caps capable of capping both 15 mL and 50 mL centrifuge tubes. Since at some stages hazardous chemicals will be centrifuged, it is necessary to have a system that protects room air quality. The buckets are loaded and capped inside the fume hood to seal in fumes before being transferred to the centrifuge outside of the fume hood. We use the vapor caps supplied by Eppendorf for the 5810 centrifuge. See Fig. 1 on p. 5.

Specific centrifuge tube adaptors are needed for each style of tube used in the centrifuge. We use adaptors supplied by Eppendorf with minor modifications.

- 15 mL plastic conical tubes – 15 mL falcon adaptors, blue and the bottom layer of the adaptor is conically formed. Holds 9 tubes per bucket; maximum of 36 tubes in the centrifuge. See Fig. 2 on p. 5.
- 50 mL plastic conical tubes – 50 mL falcon adaptors, orange with blue conical inserts that must be placed in the bottom of the adaptors before loading the tubes. The blue conical inserts must be used or the bottom of the tube may deform or split open. Holds 4 tubes per bucket; maximum of 16 tubes per centrifuge. See Fig. 3 on p. 5.
- 1 dram vials – 3-15 mL adaptors, orange, includes rubber mat at base. Assemble adaptors but only use two orange layers on top of the base (with the rubber mat). Holds 12 vials per bucket; maximum of 48 tubes per centrifuge. See Fig. 4 on p. 5.
- 15 mL glass conical tubes – 7-17 mL adaptors, gray with black rubber inserts. The adaptor holds 12 tubes per bucket, but only the two center spaces can be used with the vapor caps. Maximum of 8 tubes per centrifuge with vapor caps. See Fig. 5 & 6 on p. 5.
- 40 mL glass round bottom (and conical bottom?-test first) tubes –18-30 mL adaptors, orange with black rubber inserts. These are identical to the 50 mL orange falcon adaptors for the plastic conical tubes, but the blue plastic inserts are not used; ***you must use the black rubber inserts***. The glass tubes will break if used with the blue plastic inserts. Holds 4 tubes per bucket; maximum of 16 tubes per centrifuge. With the vapor caps: holds 2 tubes per bucket, maximum of 8 tubes per centrifuge. Do not use the white rubber mat with the black rubber inserts or the vapor caps will not fit. See Fig. 7 & 8 on p. 5.
- If the black rubber inserts used with the glass tubes become more than half-worn, they need to be replaced with new inserts. The inserts are from an old International centrifuge; they are not Eppendorf parts.

Electric hot plate and a flat-bottomed sauce pan for maintaining a boiling water bath.
Hot plate
Make: Fisher Scientific
Model #: 1152049H
Standard setting for pollen: 10/10

A vortex mixer is useful once sample volume is somewhat reduced.

Hand held engraving tool: clay must be removed from samples with much of this done using 6 µm screens. Screening speeds are significantly improved (from ~20+ minutes/sample to ~5 minutes/sample) with a hand held engraving tool used to agitate samples to facilitate migration of clay particles through the screen. We currently use a Dremel engraver model 290-01 with the carbide tip removed. Note: screening rate will vary depending upon the composition of the samples. See Fig. 9 on p. 5.

TBA warming system: TBA freezes at room temperature, making it difficult to do the needed additions. It can not be kept on a hot plate (flammable) and warm water cools off quickly. We use an inexpensive low temperature block heater (e.g. Fisher cat. # 11-718) without a block. The TBA is dispensed from a plastic bottle and the bottle sits in an aluminum foil cup set inside the block heater. See Fig. 10 on p.6.

Fume hood: all reactions must be done in a fume hood

Test tube racks: Need several for each test tube type. The polyethylene ones are better than the polysilicate which are slowly destroyed by HF

Test-tube rack that fits into the sauce pan. We take standard test tube racks and cut them in half so that they fit in our sauce pan. See Fig. 11 on p.6.

Conical centrifuge tubes:15 mL polypropylene (Nalgene #3103-0015/Fisher #05-502-10A) and 50 mL polypropylene (Falcon/BD# 2098/Fisher #14-959-49A). *Do not use polycarbonate*. Some researchers prefer to use glass tubes, switching to plastic tubes only for the HF procedure. Use the heavy duty glass 12 mL tubes, not the15 mL, as the 15 mL tubes are highly susceptible to breakage. Carefully inspect all tubes (plastic and glass) before each use for any signs of crazing, cracking, or splitting. The multiple chemical assaults weaken them over time. Minimum number needed is 20 of each type, but it is helpful to have 30-40 of each on hand.

Glassware: 100 mL graduated cylinder, 1000 mL graduated cylinder, 250 mL beakers - minimum 8 (heavy duty), larger beakers (500 or 1000 mL) for mixing solutions, several bottles for storing solutions, microscope slides and cover slips for checking samples.

Adjustable pipette and magnetic stir plate (if using LacCore Microsphere Pollen Spike suspension rather than tablets). We use an Eppendorf Research pipette that adjusts from 100-1,000 µL.

Sieve systems: We use 2 "custom" designed systems, one for greater than 80 µm screen fabrics and another for less than 20 µm fabrics. These systems are inexpensive and durable once made. Screen holder sources: For larger than 80 µm fabrics: SCIENCEWARE Mini-sieves microsieve set (BelArt #F378450000/Fisher Cat. # 14-306A); For less than 20 µm fabrics: Tupperware® bell tumblers. For nearest dealer, see the white pages of the phone book. Tupperware® styles change, but any of their large tumbler series or small storage containers with a good seal will do. Tupperware® molds their seals to match the cup rim, thus making a secure fit. Any other commercial cup and seal system that is tight would also work. See Fig. 12 & 13 on p.6.

Screen fabrics: Our current supplier is SEFAR 1-800-995-0531 their site is: www.labpak@sefaramerica.com. They sell by the yard, but the better deal is their LabPaks, which hold several 12" squares and are available in a wide range of nylon or polyester mesh sizes. The 6µm nitex screens are no longer available from SEFAR, and must instead be ordered in large rolls from Elko Filtering (http://www.elkofiltering.com).

Stir sticks: Glass stir rods or wooden applicator sticks work well. Do not use glass during the HF steps. Stirring sticks can break pollen grains if used roughly. Suspend samples by adding 2-4 mL of liquid, and then stir gently. Add remaining liquid required and stir with a "butter churn" motion - both up and down and around at the same time. We use disposable wooden stir sticks, replacing them frequently. Glass rods (except during the HF step) are fine. If a Vortex mixer is available, you may want to switch to it when pellets become small enough to suspend.

Dispensing bottles (500 mL): Squeeze bottles and repipettes are appropriate for most reagents. For the screen steps, spray bottles are helpful in moving material through the screens. Squeeze bottles are for temporary use only and not intended for long-term storage. Clean squeeze bottles immediately after use.

Vials and vial holder: We keep final samples in 1 dram shell vials with plastic closures. Do not use vials with shoulders as it makes it difficult to remove material for making counting slides. We drill out a block of wood to make a vial holder, or use a plastic vial holder. See Fig. 14 on p.6.

Cleaning supplies: washbasin, sponges, paper towels, lab wipes, detergent, glassware brushes, gloves, etc…

Benchkote for protecting the fume hood surface and countertops. This should be changed frequently to keep the area clean.

Notebook: Each preparer should have a lab notebook to log the process as it is done. The book should be a permanent bound volume, not just loose pieces of paper. We also keep a computer log. Make hardcopies of the printout, one going to each researcher that had samples processed in the batch.

Waste disposal: A bucket within secondary containment for neutralizing hazardous waste. The bucket should have a lid and needs properly completed Hazardous Waste labels. The label list the possible contents including all the chemicals used, including soda ash, and explains the daily cleanup routine. At the UMN, omitting these labels can lead to a fine. See Fig. 15 on p.6.

Equipment Images

Fig. 1: Centrifuge bucket with vapor cap.

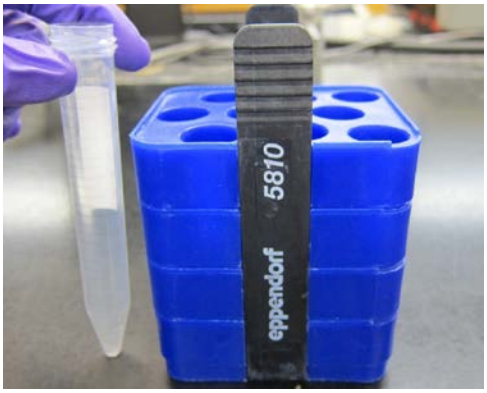

Fig. 2: Plastic 15 mL cent. tube and blue adaptor.

Fig. 3: Plastic 50 mL cent. tube and orange adaptor with blue conical insert.

Fig. 4: Glass 1 dram vials and orange adaptor in custom set up.

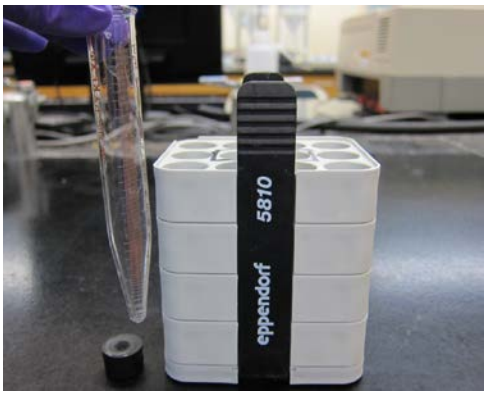

Fig. 5: Glass 15 mL cent. tube and gray adaptor with black conical rubber insert.

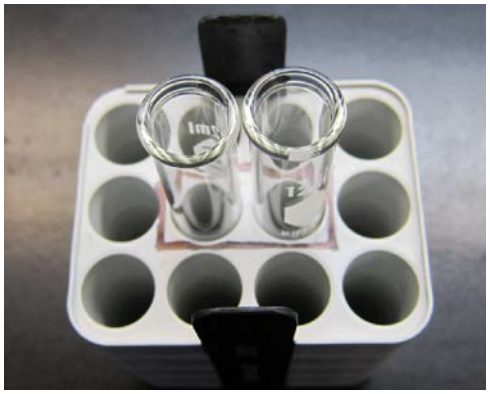
Fig. 6: The center two positions are the only ones that can be used with the vapor caps.

Fig. 7: Glass 40 mL cent. tubes and orange adaptor with black conical rubber insert.


Fig. 8: The two most central positions are the only ones that can be used with the vapor caps.

Fig. 9: Dremel hand held engraving tool.

Fig. 10: TBA warming system.

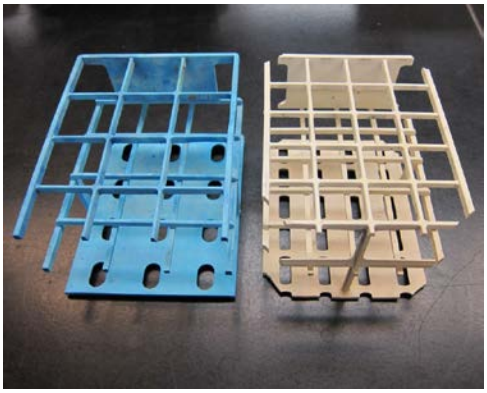
Fig. 11: Test tube racks cut down to fit inside our sauce pan.


Fig. 12: Sieve system for larger than 80 µm fabrics.


Fig. 13: Sieve system for smaller than20 µm fabrics.

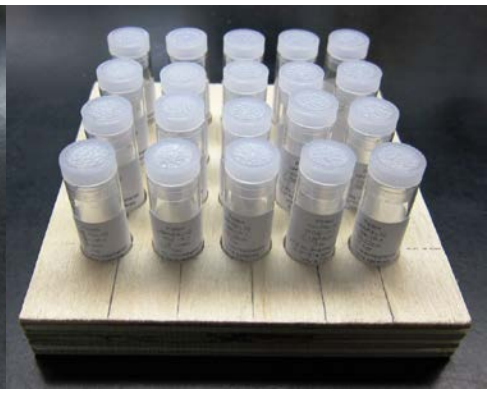
Fig. 14: 1 dram vials and vial holder.


Fig. 15: Bucket used for hazardous waste neutralization in secondary containment tub.

Procedure

Follow the steps in sequence**.** The procedure is designed so that the steps do not interfere with each other. Examples: Potassium hydroxide breaks up the sample so it screens well, carbonates and larger sand grains must be removed before the HF step to improve efficiency and prevent the formation of calcium fluoride formed from calcium carbonate and hydrofluoric acid. **Ask questions if you are not sure what to do next.**

This procedure takes between 15 and 20 hours to prepare 20 sediment samples for pollen analysis. Except where noted, it can be stopped at the end of each step. At the end of a work period, cover both the samples and the test tubes for holding the stir sticks with a paper towel. Label the test tube rack with the site name, date, your name, and the current preparation stage. Samples must be left with ~10 mL of liquid in them to prevent drying out. Leave the samples covered in the fume hood until you are able to continue the procedure.

There are 13 basic steps to the pollen procedure:
Step 1: Set-Up
Step 2: Sample preparation
Step 3: Potassium hydroxide treatment
Step 4: Screening
Step 5: Hydrochloric acid treatment
Sample Check
Step 6: Hydrofluoric acid treatment
Sample Check
Step 7: Hydrochloric rinse
Sample Check
Step 8: Acetolysis
Step 9: Dehydration
Step 10: Silicone/glycerol additions
Step 11: Record completion
Step 12: Clean up

STEP 1: Set-Up

1. Start the water bath going first - it should be boiling by the time setup is done.
2. Start spike suspension stirring, it must be stirred for a minimum of 1 hour before using. Determine the amount of spike to be added. The owner of the samples should provide this information on the Analysis Request Form (ARF). Typical 1.0 cc lacustrine sediment samples require 0.5-1.0 mL of spike ($5*10^4$ spheres/mL).
3. Set up a test tube rack with 2 sets of 15 mL test tubes labeled 1-20. One set will hold the sample; the other set will hold the stir stick for the sample in front of it. If the samples were submitted in 15 mL test tubes, use those test tubes to process the samples. Take care as you work to make sure the labels remain legible and label each sample test tube in a few different spots on the test tube. Several chemicals can remove sharpie ink.

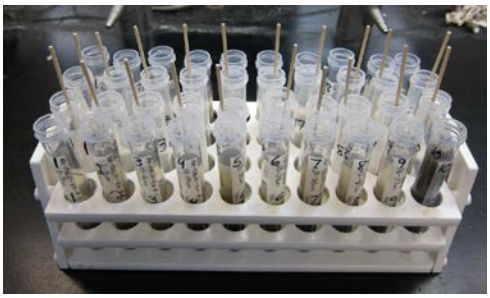

Fig. 16: Cent. tube rack set up with 20 samples and 20 test tubes holding stir sticks.

4. Set up a record log in tabular form in your lab notebook. Include in the heading: date, site name, and researcher name. The table should include columns with test tube # (1-20), core name/number, depth, sample volume, spike volume, and other notes. Keep accurate logs with each set of samples. Include dates, any errors, and anything you note of importance about the samples. You may set up your log on a

computer, but make sure to print the log and tape it in your notebook before you begin the procedure. **Write down what you do at the time that you do it.**

STEP 2: Sample Preparation

1. If the samples were submitted in containers other than 15 mL test tubes, transfer samples to test tubes, recording sample details in your notebook immediately after each transfer. This minimizes mix-ups. The samples are measured volumetrically or weighed so be sure to 100% of the sample material into the test tube using DI water. If the samples were submitted in test tubes with complete labels, be sure to label with lab IDs #1-20 in a few different spots on the test tube to prevent loss of partial labels from chemicals that can remove sharpie.
2. Add needed amount of pollen spike and 2-3 drops of TBA (tertiary butyl alcohol) to wet down any floating particles, stir, centrifuge (for 3 minutes at 3500 rpm), and decant.

NOTE ON "FLOATERS": Before every centrifuging (except those after HF, glacial acetic acid, and acetolysis), add a few drops of TBA to the test tube. TBA wets floating particles, making them easier to centrifuge. Even with this precaution, sometimes sediment particles and pollen remain floating on top of the liquid after centrifuging. Before the supernatant is poured off, check the top of the liquid for floating particles. If there are any, add a little more TBA, stir just the top, and centrifuge again at a slightly longer time setting, and a slightly higher speed setting. In extreme cases, such as in highly organic samples after the HCl steps, samples may need to be centrifuged in smaller batches (example: 1 or 2 tubes per centrifuge bucket) for periods of 15 minutes or more at 4000 rpm to sink floating particles. Be patient during this process, and remember that the sample will be lost if you decant any floating particles. Starting the procedure with a base will also help reduce the floating, as will returning to a base during the procedure.

NOTE: Decanting is the largest potential source of undesired loss of sediment. It needs to be done smoothly and directly. Watch the pellet and if there is any sign of movement, stop pouring immediately, add appropriate liquid (usually water), and centrifuge again. If the pour is too slow, you get suspension. If the pour is too fast or just an inverted dump you may lose material. DO NOT RE-START A DECANT ONCE YOU STOP IT. Centrifuge and try again. It just takes practice to get the right balance.

STEP 3: Potassium Hydroxide Treatment

This step breaks up sediment (as a detergent would) and removes humic acids.

1. Add about 6 mL of 10% KOH to each sample and stir gently.
2. Heat with occasional stirring in an actively boiling water bath for 10 to 30 minutes. Highly organic and peaty samples need more time, clays and sandy samples need less. Consult the ARF for percentage organic matter, if known.
3. Remove from heat; fill with DI water to cool, stir, add TBA, centrifuge and decant.
4. To rinse, add about 6 mL of deionized water, add a few drops of TBA, and stir. Check for clumping and color. If the sample is very dark to black or full of clumps repeat steps 1-3 after centrifuging and decanting. In very peaty/organic samples, the KOH step may need 3 or 4 repeats.
5. Repeat this rinse procedure until supernatant is transparent, not cloudy, ~3-10 times. This removes many <3µm particles that interfere with pollen counting. The number of repeats will vary with sediment composition. Record number of rinses in your lab notebook.

STEP 4: Screening

This step removes large particles and sand above 160 µm, and small particles and silt below 7 µm. This can take 1-3+ hours, if you suspect that the samples will take a long time, sieve for coarse and fine on only a few samples at a time so you can save the rest for another day. Screen size can be varied depending on the samples. We use 160 µm mesh as a standard. For very high fiber peats, you may want to screen twice - once with 180 µm and again with 160 µm to minimize trapping of pollen in the fibers. Small particles, usually silicates, less than 7 µm in size can make up a large fraction of a sample volume. When they are removed, pollen is more concentrated and the surfaces of the grains are not obscured by "snow." This step removes much of this and the remaining particles will be digested in the subsequent step with HF. The more efficiently this step is done, the more effective HF is and the less likely the need to repeat the HF step.

1. Set up coarse fabric screen holders (Fig. 1A) with appropriate mesh size. Set assembled screen on top of a 250 mL beaker and number BOTH the beaker and the screen holder with the corresponding test tube number.

Fig. 17-21: How to set up the sieves with greater than 80 µm fabric.

2. Transfer the sample onto the screen with DI water. Use a spray bottle with DI water to rinse until it appears that everything is washed through the screen except for the large particles. This usually takes 50-250 mL of water, but can take more.
3. Complete a coarse fractions sample check. Without contaminating the bottom of the screen holder, check the screen under a dissecting microscope for sediment lumps. If sediment lumps are present, rinse repeatedly until none are visible, pollen can be trapped in these lumps. If the lumps resist breaking up, recombine the entire sample, centrifuge off the water, and repeat the KOH treatment.
4. Use a spray bottle to flush remaining material on the bottom of the screen and screen holder into the beaker.
5. Discard the coarse fraction of the sample on the screen and retain what has gone through the screen
6. Set up the fine fabric sieves with 6 µm nitex fabric. Discard any screens that have irregularities such as staining, holes or stretched weaves. Make sure to put the sample number on the outside of the plastic tub.

Fig. 22-24: How to set up the sieves with less than 20 µm fabric.

7. Rinse the entire sample from the beaker into the screen. Place each screen in its own plastic tub. For very sandy samples, pour the suspension from the beaker *without stirring* onto the 7 µm screen. When pouring the last approx. 30-50 mL, swirl the beaker gently with a rotating movement, allow the sand to settle to the bottom, and then quickly pour the sample onto the screen, without dumping the sand. Heavy material, such as sand, will settle very rapidly (1 second) to the bottom of the beaker. Rinse repeatedly using 3-5 mL of water, using the same technique until only sand remains in the beaker. When the swirled material clears in about 1 second, the rinsing is complete. Discard the sand. This is very important for sandy samples because larger sand grains are not always fully dissolved by HF. They interfere with

making good slides for counting by preventing even placement of the cover slip. Additionally, the more sand removed, the more efficient the HF step becomes.

8. Rinse the fine portion of the sample through the screen. You will be keeping the portion of the sample on top of the screen and discarding the portion that passed through the screen. This step can be very tedious; to speed it up you may use the Dremel electric engraver (with the etching tip removed). Turn on the engraver to a low speed and touch it to the side of the sippy-cup. The vibration helps the water and sediment pass through the screen. Use a spray/squirt bottle to keep adding water to the sieve until clear water passes through. When using the Dremel, make sure that droplets of the sample do no splash out the top of the sippy-cup onto the counter or into other samples. Some alternatives to the electronic etcher are sonic sieving with an ultrasonic bath/probe or using a stir stick to agitate the screen. LacCore does not use sonic sieving due to concerns about damaging the bladders on coniferous pollen grains.

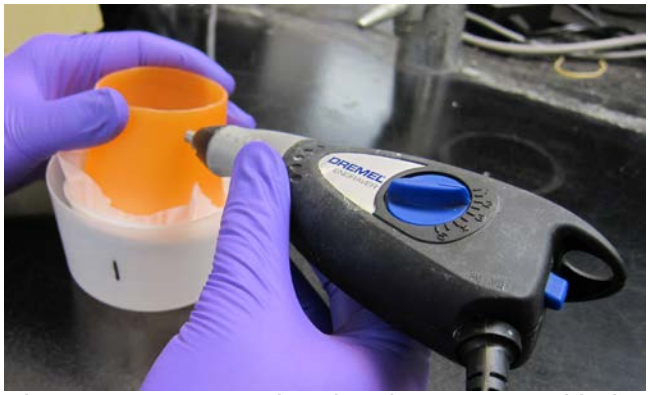

Fig. 25: How to use the electric engraver with the 7 µm fabric.

9. Transfer the >6 µm portion of the sample on top of the screen back into the 15 mL test tube using a spray bottle set to a single stream. Depending on the size of the sample remaining, you may need to fill the test tube, add TBA, centrifuge, decant, and add any remaining sample to the test tube. Carefully check the screen for any remaining material. Make sure to get the entire sample out of the edge between the screen and holder.
10. Add TBA, stir, centrifuge and decant.

Note: Nitex screens must be cleaned carefully as they can become a source of contamination. Wash first in lab detergent then soak for NO MORE THAN 5 minutes in a solution of bleach and water (approx. 10-30 cc bleach to 150-200 mL water). The bleach will dissolve the fabric if left in too long. Once screens become stained or look worn, discard them. The supply of 7 um nitex screen is limited; be careful not to forget the screens in the bleach.

Hand sieving option: Rinse the surface of the screen with a spray of DI water from a spray bottle. Then, while holding the screen over a large beaker, gently and rapidly tickle the bottom of the screen to set up a fine vibration. Do not tap hard and stretch the fabric of the screen. Repeat several times until it is apparent that no more material is passing through the mesh. You can check this by discarding the water in the beaker and then tapping some additional rinsing water through. This process is slow - up to 15-30 minutes per sample. An effective variation of this is to heat the sample in 10% sodium pyrophosphate for a couple of minutes and do the first rinsing with the same solution. If polyphosphate was used, rinse the sample 2 times after transferring the sample back into the test tube (SHMP and TBA are not miscible).

STEP 5: Hydrochloric Acid Treatment
This step removes carbonates.

1. Add about 1 mL of 10% HCl to each sample and stir very gently, especially if you expect a reaction. Control foaming with a drop or two of TBA. Add about another 5 mL, but be careful to not let the reaction overflow the test tube. If samples are still reacting after adding 6 mL of HCl, wait to put them in the hot water batch until the reaction slows, as very reactive samples can overflow during the hot water bath.

2. Heat samples for about 10-20 minutes in a gently boiling water bath. When complete, add DI water, add TBA, stir, centrifuge (use vapor caps to keep fumes out of room air), and decant. Often sample material tends to float during this procedure both before and after centrifuging. Be careful that nothing is discarded. See “NOTE ON FLOATERS” section on page 9 if "floaters" are present after centrifuging.
3. Repeat if you suspect any carbonates might still be present. This is rare, but you might want to check with the sample owner. If magnetic particles are present, repeating this step with extended boiling may help remove them.
4. Rinse twice with dH2O and a few drops of TBA.

Sample Check

Prepare a microscope slide by centrifuging samples, decanting, and adding about 0.5 to 1 mL of DI water. Use a Pasteur pipette with a bulb to suspend the sample, then transfer one drop to a microscope slide. Add a cover slip and check at 250x. If you see crystalline material or “snow” of 0.5-3 µm particles near the resolution of the microscope, you have silicates. If you see nothing but brown organic-looking particles, then silica is not a problem, often you will get a mix.

STEP 6: Hydrofluoric Acid Treatment

This step removes silicates (sand, silt, clay). HF dissolves glass; do not use glass rods, test tubes, or slides. Samples with silica (sand/silt/clay) content will require hydrofluoric acid treatment. If you saw ANY sand during the screening step or sample check, complete the hydrofluoric acid treatment.

Fig. 26: Appropriate stir **Wear under**

Wear long plastic apron with full sleeves so both arms and legs are covered. both splash goggles and the full face shield and double glove with nitrile rubber. Although HF is dangerous, the risk is entirely manageable if the safety precautions are followed. Review the safety section at the start of this document.

1. Place the samples in the rack for the water bath. Do not pick up individual samples again until they are transferred to the centrifuge. Add about 6 mL of 48% HF to each sample using the dispenser. Avoid any drips. Leave the wooden stir sticks in the test tubes. Stir carefully.
2. Transfer rack to actively boiling water bath. Heat in the actively boiling water bath for 20 minutes, stirring once at the half-way point. When done, remove rack from the water bath.
3. Fill with 95% EtOH (ethanol) to cool (**NO TBA**), centrifuge (use vapor caps to keep fumes out of room air) and decant into waste container.

Note: the wood stir sticks frequently break while in the HF, but it is too dangerous to take them in and out for each stirring. If a stick breaks, leave the broken piece in the test tube until one HCl rinse is completed. It can then be pulled out safely. A good method to do this is to take a fresh stir stick, line it up parallel to the broken piece, and then slowly pull the good one out. The broken piece will adhere to the good piece by cohesion.

Note: “Floaters” also often occur after the HF boil. If this happens, follow the “NOTE ON FLOATERS” section of page 8, but add 95% EtOH instead of TBA.

Fig. 27: Double glove 10% HCl as liquid on

It is best not to stop work immediately after this step. If you must, add to the samples to keep them moist or do as much of the next procedure possible. Do not add water as this causes excessive clumping.

Heavy liquid option: LacCore uses lithium sodium tungsate (LST) heavy samples with very high percentages of silt/clay/sand, where repeated HF treatments may not be enough to remove silicates. Please contact LacCore scientific staff for more information on LST use.

Sample Check
Examine the sediment packed in the bottom of the tubes after the last rinse. If you see a small separate gray/white layer, there is still clay/silt in the sample, repeat the HF step. Do not do the HF step for more than a total of 40 minutes. HF can start to degrade the pollen grains after this.

PHOTO

Fig. 28: Sample showing the separate gray/white layer.

STEP 7: Hydrochloric Acid Rinse
This step is directly related to the HF treatment. It breaks up siliceous colloidal clumps that formed during the silica digestion.

1. Add about 6 mL 10% HCl. Stir gently and heat in water bath for about 3 minutes, allowing the sample to get hot. Add DI water, add TBA, stir, centrifuge (use vapor caps) and decant into waste bucket. Remember to check for "floaters" and take appropriate steps if necessary.
2. Repeat the DI water rinse twice.

Sample Check
Set up a microscope slide, as in the previous sample check. Apply a cover slip to each sample. If the cover slip will not lie flat because of grit, then the HF/HCl steps need repeating. Examine under a binocular microscope at 250x. Look for crystalline materials. If present in sufficient quantity to interfere with pollen counting, then the HF/HCl steps need repeating. Also, look for large grayish clumps. They look a bit like dirty rain clouds. These colloidal clumps were not broken up by the HCl treatment (Step 7). If they are present, then repeating the HF/HCl step is needed, but only 10 minutes of hot HF is sufficient. Do not do the HF step for more than a total of 40 minutes. Some samples may need to be rescreened with 7 um mesh to remove more silicates if the maximum number of HF steps has been completed. If the pour-off and nitex steps were done carefully, there is rarely a need to repeat the HF step. In 99% of the cases, 2 HF treatments will remove enough silicates to make the samples countable.

STEP 8: Acetolysis
This step removes some organic matter, cleans the surface of the pollen grains, and stains the pollen grains a golden brown. This entire procedure takes about 1 hour and must be done efficiently and with no interruptions. The water bath needs to be full and ACTIVELY boiling during the actual acetolysis treatment. Do not start until it is. Acetolysis is only effective near 100°C. All reagents are hazardous and the acetolysis mixture reacts explosively with water. Be sure waste bucket has additional soda ash because the glacial acetic acid reacts for quite a long time. You may need to keep adding soda ash to the bucket. Neutralize and wash the squeeze bottle used for applying the 9:1 mixture. Air dry completely, tape the screw cap to the side of the bottle as condensation may form if the cap is screwed on, label the bottle with labeling tape as "Clean", and set aside for next user.

1. Add ~6 mL glacial acetic acid, stir, centrifuge (use vapor caps) and decant; repeat this step one more time. This step removes water from the samples.
2. Prepare a 9:1 conc. acetic anhydride : conc. sulfuric acid mixture in a 100 mL graduated cylinder. Prepare this fresh while the samples are centrifuging after the second glacial acetic acid rinse. Both reagents are hazardous. Carefully pour 90 mL of acetic anhydride (as it comes from the bottle) into a *dry* 100 mL cylinder. The bottom of the meniscus should be touching the 90 mL line. Add 10 mL of conc. sulfuric acid (as it comes from the bottle) slowly until the bottom of the meniscus touches the 100 mL line. You do not need to stir as the acid sinks into the anhydride. The reaction is exothermic, and the cylinder will get quite warm. If you add too much sulfuric acid (over 11 mL), the mixture turns brown. Discard into the waste bucket (SLOWLY - remember it reacts violently with water, and there is water in there). Then start again.
3. Add ~5 mL 9:1 mix acetic anhydride : sulfuric acid, stir, and boil for *exactly* 2 minutes. Add 1 mL glacial acetic acid (*not TBA*), stir, centrifuge and decant. Timing is very important; do not boil for more than 2 minutes. This step should be done with no delays. The samples must not be in the acetolysis mixture for longer than necessary.
4. Add ~6 mL glacial acetic acid, stir, centrifuge and decant.
5. Rinse 3 times with DI water and a few drops of TBA.

Sample Check
Repeat the procedure for making quick microscope mounts only if something in the previous check suggested there might be problems such as excessive clumping or too many small particles or abundant pyrite. Extra treatments should only be done if the pollen counting would be simplified, as extra steps are time-consuming.

Un-clumping: This is needed if there are many clumps visible when the sample is stirred or if a lot were seen under the microscope. A few do not matter as they can be dispersed when stirring in the silicone oil at the end. Two alternatives are available. (1) Repeat the hot 10% KOH treatment (Step 2), heating for 20 minutes followed by water rinses; or (2) Follow the hot 10% KOH procedure, but substitute 10% sodium pyrophosphate. In extreme cases, try both. Rinse 2x with DI after either procedure.

Pyrite (or other iron sulfides) removal: If the sample appears very black when stirred, it may be excessive pyrites. Under the microscope these look like tiny black cubes about 1-4 µm on a side. Nitexing is the removal method of choice, but sometimes not enough crystals pass through. In addition, the crystals can cluster in large (10-100 µm) spherical clumps that look like black balls which will sometimes break up under pressure of a wooden stick on the surface of the cover slip. They can be removed with nitric acid, but the acid is also corrosive to pollen, so great care must be used. We just live with the pyrites.

STEP 9: Dehydration
This removes water, which does not mix with the silicone oil mounting medium. If the water is not 100% removed, irreversible clumping always occurs. The sample will have to be discarded, and a new one prepared. Do this step and the next one in sequence. Do not start unless you can finish both of them.

1. Add ~6 mL 95% EtOH, stir, centrifuge and decant.
2. Add ~6 mL 100% EtOH, stir, centrifuge and decant.
3. Add ~6 mL TBA, stir, centrifuge and decant.
4. Transfer samples into numbered 1 dram vials with TBA, centrifuge and decant. When transferring, it is ESSENTIAL that the correct sample goes into the correct vial. Hold the vial up and read the number on it, then hold up the test tube, read the number on it and then read the corresponding information in your notebook.
5. In order to transfer all of the material from the test tube to the vial, you may need to centrifuge and decant the material in the vial one or more time. Keep the TBA warm during this process.

STEP 10: Silicone/glycerol oil addition
This is the final microscope slide study medium.

1. Add enough silicone oil or glycerol to cover the sample, usually 3-15 drops.
2. Stir samples very thoroughly with a clean stir stick. This is to ensure that all the particles will be coated with silicone oil. Be sure to get everything on the sides of the vial. If all particles are not coated you will have to discard the sample and repeat the entire process again
3. Cover the open samples in the vial rack by folding a paper towel over everything so that the TBA can evaporate. Do not cap them or wrap with plastic wrap. Place in the fume hood to increase the evaporation rate. *Do not let the vials go uncapped for more than 24 hours, if this happens the dehydration and oil steps need to be repeated.*
4. After the TBA is evaporated, the samples should be checked again for oil volume. Ideally, about 1-2 mm of clear oil should sit above the sample.
5. Add vial labels (See labeling note and example on page 14).

If too much is added, then some will need to be removed - a very tedious process. If too little is added the particles of processed sediment will not be adequately coated with the oil, and the sample will dry out. This means discarding the sample and repeating the entire process again. Here are some guidelines.
Samples that had high clay will need less than peat or organic lake mud samples. If there is just a film of material on the bottom of the vial, then 3 drops is plenty. If there is 1-2 mm of sediment, then start with 5-8 drops. After stirring, you may want more.

Excess oil can be removed by transferring the sample back into a test tube with TBA, and rinsing several times with TBA. Care is needed on the first rinse because the oil will greatly increase the density of the TBA oil mix making the centrifuging less effective. At the other extreme, samples with too little oil may sometimes gel almost into a rubber and are hard to re-suspend after 6 months. When stirring a bulky sample after the TBA is gone, the sample should feel only a bit more viscous than the oil alone.

Labeling: Make sure all labels are in the same format, clearly displayed, and easy to read. Enter the label information into a spreadsheet, import the spreadsheet into the DYMO LabelWriter software, and print the labels for a batch altogether. Below, two labels are shown. The left label shows the names of the fields, and the right label is an example of a label from a completed sample.

MATERIAL material medium Sample ID Depth Sample

POLLEN in silicone oil FERR05-1A- 6B-1P-1W Depth: 7.0-8.0 cm

STEP 11: Record Completion

Make sure that everything is properly complete in your own lab book. Your book is sometimes checked for details on a procedure done to a specific sample, sometimes years later.

Transcribe your log into the FileMaker Pro pollen log. Record any errors or discarded samples. Be certain the sample owner is aware of any problems; e-mail them to make them aware of any problems and the reasons for such problems. **Do not wait for the sample owner to get a copy of the log and ask about the problems, contact them first and cc lab manager.** Print a copy of the FileMaker Pro pollen log entry and give it to the sample owner with their samples. Do not begin another batch until this is done. It is easier to find samples in this log if it is in chronological order.

Update the billing database with the number of samples processed for each project code.

STEP 12: Clean Up

Laboratory glassware: Wash every item after use. Wash with lab detergent and bleach, rinse 3 times with tap water followed by 2 DI water rinses. Gloves and plastic apron are recommended when washing glassware. The glassware should have no spots or rings when dry. Put the dishes away when they are dry.

Household bleach oxidizes pollen. The 15 mL and 50 mL test tubes must be soaked in straight bleach for at least a few hours between sample sets. Wash them, place them in soaking jars overnight, and then do a final rinse in low purity DI water.

Soak plastic sieving supplies (sippy-cups, rings, etc.) and spray bottle nozzles in straight bleach periodically.

Nitex screens must be cleaned carefully as they can become a source of contamination. Wash first in lab detergent then soak for NO MORE THAN 5 minutes in a solution of bleach and water (approx. 10-30 cc bleach to 150-200 mL water). The bleach will dissolve the fabric if left in too long. Once screens become stained or look worn discard them. Nitex screens are expensive, and often take a long time to order, so take care of them and don’t forget them in the bleach.

Countertops, shelves, hood surface: Clean regularly with bleach water (1-2 capfuls of bleach:1 full spray bottle of water). Hood surface and countertops after each use and shelves at least once a month. Airborne dust contains "foreign" pollen. Place Benchkote on the surface of the fume hood and change it often.

Since the acids and bases used are inorganic, they may be disposed of down the drain after they have been neutralized (pH $\geq$ 7) in the waste bucket. All decanting involving hazardous chemicals was done into the waste bucket. Neutralize it with a scoop or two of soda ash as needed throughout the day. Foaming can be controlled by squirting the reaction with 95% EtOH (ethanol) or TBA (tertiary butyl alcohol). ALWAYS completely react wastes in the waste bucket by adding more soda ash until they test neutral or slightly basic using pH tape. DO NOT leave un-reacted wastes in the bucket overnight. Clean it for the next person. Hint: it can sometimes take 10-15 minutes to clean up properly. Plan your time accordingly. Dried soda ash on the counters is considered mishandled hazardous waste. The fume hood surface must be kept clean and cleaned up at the end of the night. At the UMN, leaving dried chemicals on the fume hood surface can lead to a fine.

Document history

This SOP is based on a long line of sequentially modified versions of the pollen preparation procedure of Dr. E. J. Cushing (UMN, Dept. of Ecology, Evolution, and Behavior, St. Paul, MN 55108) as recorded in 1977. Modification sequence as follows: L.C.K. Shane (Nov. 1981), L.C.K. Shane and G. A. King (July 1985), L.C.K. Shane, (June 1986, Oct. 1992, Nov. 1998, June 2002), Jessica Heck (Dec. 2010), photos Christa Drake/Rob Lusteck (Dec 2010), Jessica Heck (Dec. 2012), Amanda Yourd (2014).

This procedure is ultimately a modification of that described by Faegri and Iverson (1975)

Faegri, K. & Iverson, J. 1975: Textbook of Pollen Analysis. 295 pp. Hafner Press, New York. (Note: newer editions have been published.)

Pollen Preparation Procedure, summary

Important Notes

Lab coats, chemical goggles and gloves must be worn at all times. Many of these chemicals can be very dangerous and this document is not intended to train you on safety. Additional safety information is found in the Art of Pollen and the chemical Safety Data Sheets (SDS). It is assumed that you have read the whole pollen SOP, centrifuge SOP, and chemical SDS before starting for details on set up, record keeping, safety, equipment use and tailoring this process for sample composition.

Procedure

1) Fill and start the hot water bath (90+ deg. C).
2) Ideally samples were submitted in screw cap 15 mL centrifuge tubes, add ~6 mL high purity deionized water (dH20). If not, transfer a batch of 20 samples to 15 mL plastic centrifuge tubes using dH2O, there should be a minimum of 6 mL dH20 in each tube before continuing.
3) Add the required amount of spike. The spike should be on the stir plate for one hour before use and should remain stirring as it is added to the samples. For a typical lacustrine sediment sample 1cc in size, 0.5-1.0 mL of spike is required. Add a few drops of tert-butyl alcohol (TBA) and stir with a wooden stir stick. Centrifuge the samples at (2400 rcf) 3500 rpm for a minimum of 3 minutes then decant the supernatant off of the pellet.
4) Add ~6 mL of 10% potassium hydroxide to each centrifuge tube. Stir with a wooden stir stick and boil for 10-30 minutes, stirring halfway. Add dH2O, add few drops of TBA, centrifuge and decant. Add ~6 mL of deionized water, add a few drops of TBA, stir, centrifuge and decant; repeat this rinse procedure until supernatant is transparent.
5) Sieve with 160 µm screens, retain <160 µm portion and sieve through a 6µm sieve. Retain the >6µm portion and transfer it back to a 15 mL centrifuge tube. Add a few drops of TBA, stir, centrifuge and decant.
6) Add ~6 mL of 10% hydrochloric acid (HCl) to each centrifuge tube. Stir with a wooden stir stick and boil for 10-20 minutes, stirring halfway. Add dH2O, add a few drops of TBA, stir, centrifuge and decant. Add ~6 mL of deionized water, add a few drops of TBA, stir, centrifuge and decant; repeat rinse twice.
7) Add ~6 mL of hydrofluoric acid to each centrifuge tube. High health HAZARD. Additional personal protective gear required, see Art of Pollen for details. Stir with a wooden stir stick and boil for 20 minutes, stirring halfway. Add a few mL of 95% ethanol (EtOH), stir, centrifuge and decant.
8) Add ~6 mL of 10% HCl to each centrifuge tube. Stir with a wooden stir stick and boil for 3 minutes. Add dH20, add a few drops of TBA, stir, centrifuge and decant. Add ~6 mL of deionized water, add a few drops of TBA, stir, centrifuge and decant; repeat rinse twice.
9) Add ~6 mL glacial acetic acid, stir, centrifuge and decant; repeat this step one more time.
10) Add ~5 mL 9:1 mix acetic anhydride : sulfuric acid, boil 2 minutes. Add 1 mL glacial acetic acid, stir, centrifuge and decant. Timing is very important; do not boil for more than 2 minutes.
11) Add ~6 mL glacial acetic acid, stir, centrifuge and decant.
12) Rinse 3 times with DI (don't forget TBA).
13) Add ~6 mL 95% EtOH, stir, centrifuge and decant.
14) Add ~6 mL 100% EtOH, stir, centrifuge and decant.
15) Add ~6 mL TBA, stir, centrifuge and decant.
16) Transfer samples to 1 dram vials with TBA, centrifuge and decant. Add enough silicone oil or glycerol to cover the sample, stir. Add vial labels. Allow TBA to evaporate for up to 24 hours; cap the vials.