Procedures for Extracting Pollen from Lacustrine Sediments – **Detailed information**

Modified from LacCore's SOP, for application in lab in 217 Pacific Hall, Univ of Oregon
http://lrc.geo.umn.edu/laccore/

Items in yellow highlight need checking

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.

The method described bel ow is suitable for lacustrine sediments containing varying proportions of organic material, sand, silt, and clay. It is a detailed outline of the techniques developed by L.C. Cwynar which incorporate methods devised by K. Faegri and J. Iversen.

Original citations for methods:

- "Laboratory Technique" in Faegri and Iversen 's Textbook of Pollen Analysis (1975, Hafner Press, 3rd edition)
- L.C. Cwynar , E. Burden, J.H. McAndrews 1979 , "An inexpensive sieving method for concentrating pollen and spores from fi ne-grained sediments," Canadian Journal of Earth Sciences 16, 1115-1120.

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 and gloves must be worn at all times. Chemical splash goggles must be worn during most steps of the process. You must wear closed toe shoes and long pants. If you have long hair, make sure to tie it back.

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 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.

A chemically resistant vinyl apron with full-length sleeves, face shield (in addition to glasses or goggles), and rubber gloves over nitrile gloves must be worn when using hydrofluoric acid (HF). 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 (sodium carbonate) 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 heating block.

DI water or dH_2O: water from the Barstead benchtop ultrapure water filter. Turn to "on" and wait until unit reads "18.2" before dispensing. When done using, set to "standby".

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.

Use the pollen processing data sheet to keep notes on anything notable during processing, sample volume, number of exotic tablets, dates, etc. Place a scan of this data sheet on the Paleo file server and keep the original in the three-ring binder for your site. 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, sample depth, sample volume, preparation date. Make sure to double check that the label matches what you have written in your lab notebook. The bottom of the glass vial holding the final sample can be etched with a glass engraver pen with the site code and sample depth. 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 at least one liter 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.

- For the IEC HN-SII centrifuge in the hood: use max rpm (about 2000 rpm) for a full ten minutes.
- For the Fisher Scientific centrifuge on the bench: Use 2500 rpm for five minutes.

Since at some stages hazardous chemicals will be centrifuged, it is necessary to have a system that protects room air quality. For steps that involve HF and Acetolysis, use the centrifuge located within the fume hood. Cap the tubes during these steps. For other steps, the tubes are

capped inside the fume hood to seal in fumes before being transferred to the centrifuge outside of the fume hood.

Specific centrifuge tube adaptors are needed for each style of tube used in the centrifuge.

- 15 mL plastic conical tubes –
- 50 mL plastic conical tubes –
- _1 dram vials –

Heating block: Make: ---
Model #: 1152049H
Standard setting for pollen: 10/10

A vortex mixer is useful when there is about 4 ml of liquid in 15 ml tubes, but should be used with care when sample is large (ca. 1 ml). After HF step, manual stirring with stir rods may be entirely substituted with the vortex mixer.

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.

TBA warming system: TBA freezes at room temperature, making it difficult to do the needed additions. It cannot 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.

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

Test tube racks: Need several for each test tube type.

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.

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: The 7 µm nitex screens may be ordered in large rolls from Elko Filtering (http://www.elkofiltering.com).

Vortex and stirring: Copper rods, 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. When adding water or chemicals, first suspend samples by adding 2-4 mL of liquid, and then stir gently using vortex mixer. If not breaking apart and suspending in the sediment, use the stir rod, then vortex. Usually, stir rods are needed until the HF step removes the sticky clays. Add remaining liquid required and stir with a "butter churn" motion - both up and down and around at the same time. Do not vortex if more than 12 ml in the 15 ml tube. Incomplete mixing of sample is a major reason for poor results.

Dispensing bottles (500 mL): Squeeze bottles 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 large "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.

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:
Water, KOH, HCl, and Alcohols may be neutralized in a bucket with sodium carbonate and poured down the drain.

HF and Acetolysis mixture must be placed in a waste bottle for pick-up by Environmental Health and Safety. The bottles must be labeled correctly with tags.

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 12 and 15 hours to prepare 12 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 and load tubes
Step 2: Potassium hydroxide treatment and large-fraction screening
Step 3: Hydrochloric acid treatment
Step 4: Hydrofluoric acid treatments
Step 5: Hydrochloric acid rinse
Step 6: Acetolysis
Step 7: Sodium pyrophosphate (NaPyrP) and 7-micron Nitex sieving.
Step 8: Staining and Dehydration
Step 9: Silicone addition
Step 10: Record completion
Step 11: Clean up

STEP 1: Set-Up and load tubes

1. Start the heating block.

2. Start data sheet and lab notebook entry. Sample size and consequently volume of chemicals used in treatment will vary, depending on the concentration of pollen within the sediment. The following procedures are based on a sample size of 1 ml or 1 cubic centimeter. For highly organic samples (>50% organic matter), you may wish to use only 0.5 ml, and thus can greatly save the amount of time and chemical needed to process the sample.

3. Set up a test tube rack with two sets of 15 mL test tubes labeled 1-12. The tubes should have been cleaned in bleach after the previous batch. One set will hold the sample; the other set will hold the stir stick for the sample in front of it. 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.

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.**

5. Add to tubes needed amount of pollen spike (1 to 3 lycopodium tablets). Add about 2 ml of HCl and let it dissolve about 10 min. Note exotic batch number on data sheet.

6. Add sample. The samples are measured volumetrically so be sure to 100% of the sample material into the test tube using DI water. Measure volumes using syringe or brass samplers and place in numbered tubes. Clean sampler carefully between samples.

7. Fill with water to about 10 ml, and 2-3 drops of TBA (tertiary butyl alcohol) to wet down any floating particles, stir, centrifuge, 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 2: Potassium Hydroxide Treatment and Screening

This step breaks up sediment (as a detergent would) and removes humic acids. It is followed by screening out large particles and water rinses.

1. Add about 8 mL of 10% KOH to each sample and stir gently.
2. Heat with occasional stirring in at 90°C for 10 minutes. Highly organic and peaty samples need more time, clays and sandy samples need less.

3. Remove from heat; fill with DI water to cool, stir.

4. Sieve samples with 200 μm or 250 μm sieves (No. 70) using dH_2O into plastic beakers. Save macrofossils if present. Use a squirt bottle with dH_2O 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. 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.

5. Return to 50 ml tubes (this could require several centrifuge steps. Keep beaker with sample covered with paper towels as you centrifuge water. Repeat SCD until supernatant is fairly clear (very light tea color). [See note at end of procedure if there is coarse sand in the sample].

6. Return sample to 15 ml tubes. Repeat KOH step if supernatant remains yellow, but always follow KOH step by several water rinses.

STEP 3: Hydrochloric Acid Treatment
This step removes carbonates.

Any reaction of HCl with the sample should be noted because the presence of carbonates can result in erroneous radiocarbon dates and carbonate content may be quantified using other procedures.

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 the heat block. 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 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.

STEP 4: Hydrofluoric Acid Treatment
This step is needed to remove diatoms and silicates (sand, silt, clay), which are present in nearly all lake sediments. Skip if the sample is mostly peat. HF dissolves glass; do not use glass stir rods, test tubes, or slides.

Wear long plastic apron with full sleeves so both arms and legs are covered. Wear both splash goggles and the full face shield and double glove with nitrile under rubber. Use wood stick applicators.

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 heating block. Do not pick up individual samples again until they are transferred to the centrifuge. Add about 8 mL of 48% HF to each sample using the dispenser. Avoid any drips. Leave the stir sticks in the test tubes. Stir carefully.

2. Heat at 90°C bath for 20 minutes, stirring once at the half-way point. When done, remove rack from the water bath.

3. Cap, centrifuge in the hood, and decant into waste container.

4. Repeat HF step if necessary, or do a cold HF step overnight (e.g., for sandy samples).

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.

It is best not to stop work immediately after this step. If you must, add 10% HCl to the samples to keep them moist or do as much of the next procedure as possible. Do not add water as this causes excessive clumping.

Heavy liquid option: LacCore uses lithium sodium tungsate (LST) heavy liquid on 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.

STEP 5: 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 5). 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 screened 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 6: 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 heat block needs to be at 95°C 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. Using the repipete dispensers on the sulfuric acid and acetic anhydride bottles. Using a small **dry** glass beaker, add 4 ml acetic anhydride and 0.5 ml sulfuric acid. Both reagents are hazardous. You do not need to stir as the acid sinks into the anhydride. The reaction is exothermic, and the beaker will get quite warm.
3. Add to tube, stir, and place in heat block for *exactly* 3 minutes. (Mix and add mixture every 30 seconds for six tubes). Then, add a few mL glacial acetic acid (*not TBA*), stir, centrifuge and decant. Timing is very important; do not heat for more than 3 minutes. This step should be done with no delays. The samples must not be in the acetolysis mixture for longer than necessary. Repeat for the second set of six tubes.
4. Add ~8 mL glacial acetic acid, stir, centrifuge and decant. (to remove remaining acetolysis)
5. Rinse with DI water and a few drops of TBA (if needed), stir, centrifuge, decant.

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) Use warm 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. It is best to leave them in the sample.

STEP 7: Sodium pyrophosphate (NaPyrP) and 7-micron Nitex sieving.
This removes the fine clay fraction. The entire step may be skipped if samples are highly organic.

1. Make warm NaPyrP (5%) by heating on hot plate in glass beaker.
2. Add 8 ml warm NaPyrP and stir.
3. Set up Nitex sieves by placing mesh within threaded tubes. Sieve using warm NaPyrP. Use the dremel tool to coerce through the mesh.
4. Wash sample from nitex into a clean beaker or back into its test tube with DI water. **Centrifuge for 10 min** and decant.
5. Wash with DI water, stir, centrifuge, and decant.

Details: 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 screen holder. 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.

Transfer the >7 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.

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.

STEP 8: Staining and Dehydration

This removes water, which does not mix with the silicone oil mounting medium. Do this step and the next one in sequence. Do not start unless you can finish both of them. It is important to completely dehydrate the residue so that pollen will not swell and eventually rupture. If the water is not 100% removed, irreversible clumping always occurs.

1. Add 1 drop of 0.1% safranin stain, stir, and wait for 2 min. Note that safranin in dropper should be a light red color…and nearly transluscent…not dark red and opaque. Otherwise, the pollen will be overstained.
2. Add ~6 mL 95% EtOH, stir, centrifuge and decant.
3. Add ~6 mL 100% EtOH, stir, centrifuge and decant.
4. Add ~6 mL TBA, stir, centrifuge and decant.
5. Transfer samples into numbered 1 dram (5 ml) vials with TBA, centrifuge and decant. Use plastic vials in centrifuge tube shields to support glass vials in the centrifuge shields. Etch sample name on the base of the vial. Add sample label to vial.

   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.
6. 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 9: Silicone oil addition

This is the final microscope slide study medium.

1. Add enough silicone oil 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.

If too much oil 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.

For removing coarse sand remaining after the coarse (200 micron) sieving:
For very sandy samples, pour the suspension from the beaker *without stirring* from the plastic beaker into the 50 ml tube. Swirl the beaker gently with a rotating movement, allow the sand to settle to the bottom, and then quickly pour the sample onto the tube, 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.

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.

STEP 10: 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 HCl and KOH used are inorganic, they may be disposed of down the drain after they have been neutralized (pH > 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.

Chemicals safe for stopping overnight or longer:	**Chemicals not safe for pollen for extended periods:**
HF (but in fume hood, with fume hood ON)	HCl
Water	KOH
GAA	Acetolysis (under 3 minutes).
Ethanol	
TBA	
Sodium pyrophosphate	

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.)

It was then modified by Dan Gavin for use at the University of Oregon.

To make a slide, thoroughly mix pollen residue in sample vial with a wooden applicator stick. If the concentration of residue is great place a small drop of silicon oil on a labeled glass slide. Add a small amount of pollen residue mixing thoroughly with silicon drop.If the pollen residue is slight, it can be applied directly to the slide. Place cover slip on sample and let sit until silicon oil has dispersed across the cover slip. Place a small drop of nail polish on each corner of the cover slip to prevent it from moving during counting.

The amount of residue and silicon oil placed on the slide should be great enough to completely fill the cover slip. Too much oil will ooze onto cover slip; too little oil will result in large air bubbles under cover slip. To ensure even distribution of pollen, it is best to tr- ace a small "X" on the slide with the pollen residue, placing small dabs of residue between the "arms" of the "X."

Studies have shown that pollen spreads differentially under the cover slip (for example, small , light pollen grains tend to move towards the edge of the cover slip). Thus, when counting, it is important to count traverses spaced evenly across the entire slide. This is particularly important in samples with dense pollen concentrations.