THE ART OF POLLEN PREPARATION

GENERAL INFORMATION

This description is designed (1) as a source of general information about pollen processing procedures used at the Limnological Research Center (LRC), University of Minnesota, and (2) as a very detailed training document for undergraduate assistants who may have had limited laboratory experience. It is another in 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. 9, 1981
L.C.K. Shane and G. A. King	July 12, 1985
L.C.K. Shane	June 26, 1986
L.C.K. Shane	Oct. 21, 1992
L.C.K. Shane	Nov. 5, 1998
L.C.K. Shane	June 7, 2002

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.)

GENERAL PROCEDURAL RECOMMENDATIONS

SPECIAL NOTE TO STUDENT TECHNICIANS

The following recommendations regard sound laboratory techniques, safety practices, and manners. YOU ARE RESPONSIBLE FOR FOLLOWING THESE PROCEDURES.

1. SAFETY REQUIREMENTS: The U MN requires annual, documented (complete and file form), basic laboratory safety training. This is obtained by reviewing prescribed tapes or attending annual training sessions provided the Department of Geology. Remember at all times that you are handling strong acids and bases. These chemicals, the glassware, and the machinery are potentially hazardous.

a. Safety clothing: Eye protection and clothing protection must be worn at all times. Lab coats, aprons, gowns, goggles, faceshields, and gloves are available. Wear shoes that cover your feet. Open sandals are not allowed. For most pollen processing procedures, personal eyeglasses or plastic safety glasses and a lab apron are sufficient. Avoid contact lenses because chemicals can be trapped under a lens. HF and acetolysis procedures require more extensive protection. See these steps for details.

b. Fume hood: All reactions must be done in a fume hood.

c. Waste disposal: Since the acids and bases used are inorganic, they may be disposed of down the drain after reacting them in a waste bucket placed in the hood and holding ca. 1 scoop of soda ash (cheap grade of sodium bicarbonate - purchased in 50 LB bags). Foaming can be controlled by squirting the reaction with 95% EtOH (ethanol) or TBA (tertiary butyl alcohol). ALWAYS completely react wastes in the waste buck by adding more soda ash until they test neutral or slightly basic using pH tape. DO NOT leave unreacted 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.

Note on the waste bucket: The bucket should be contained in a larger bucket in case of a spill or foaming overflow. We use the bottom portion of a 1 gallon plastic bleach bottle for the inner bucket and another larger plastic outer container. The outer and inner container need properly completed Hazardous Waste labels. Ours list the possible contents including soda ash and the various components and explain the daily cleanup routine. At the U MN, omitting these labels can lead to a fine paid out of the investigator's budget.

d. Hot plate and water bath: UNPLUG and 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).

2. PROCESSING LOG RECORDS: You will have a notebook to log each batch of pollen samples and each step in the batch as it is done. Keep accurate logs with each set of samples. Include dates, any errors, and anything you note important to the samples***, Write down what you do at the time you do it.*** When finished, enter the information into the Pollen Processing log file on the main lab computer. Print two copies, putting one in the lab log ring binder book and giving one to the owner of the samples. Each log must have the following information for each sample: 1) site name and location; 2) core #; 3) sample depth; 4) sample volume; 5) spike amount and batch; and 6) any other information that is specific to those samples.

3. LABELING: 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. Write clearly: ten years from now someone may need to read the vials and figure out what is in them. Additionally, during processing, it is critically important to label all tubes and beakers correctly to minimize errors related to mixing of samples.

4. ERRORS: 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 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 simply start over. In almost all cases, there is material to replace what is lost or contaminated.

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.

ASK QUESTIONS if you are not sure what to do next.

5. 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.

6. FOLLOW THE STEPS IN SEQUENCE: The procedure is designed so that the steps do not interfere with each other. Examples: KOH breaks up the sample so it screens well; carbonates and larger sand grains must be removed before the HF step or the sample will be gritty from either or both unreacted sand grains and CaF_2 formed from calcium carbonate and HF.

7. OPERATING THE CENTRIFUGE: With the IEC model K centrifuges, usually a setting of 30 for 4-5 minutes is sufficient. Use swinging bucket heads (fixed heads do not work for pollen). It is essential that the shields and trunion rings be balanced. Weights are engraved on each part. The centrifuge tubes with the samples must be filled to the same height. Constant careless use will wear out a centrifuge unnecessarily. Use your ears - if it sounds strange, turn off the machine and check for balancing.

8. CONTAMINATION PROBLEMS: These come from poor housekeeping. The labs and glassware must be kept clean.

a. Laboratory glassware:

Do dishes regularly. Wash every item after use. Remove all tape and any numbers in wax pencil or magic marker. Use bleach as described below. Wash with lab detergent, rinse 3 x with tap water followed by 2 DI water rinses. Glassware should have no spots or rings when dry. Do NOT rely on simple rinsing. Put away immediately when dry. See also notes on reagents.

Household bleach oxidizes pollen. The 15 and 50 ml test tubes must be soaked in straight bleach for at least a few hours between sample sets. Wash them first, then place in soaking jars overnight, then do final rinsing. Also add a small amount of bleach to water where beakers and screens are soaking before cleanup. Do not leave brushes in bleach - it destroys them. Gloves and apron are recommended when washing glassware.

b. Countertops, shelves, hood surface: Clean regularly. Hood surface and countertops after each use; shelves at least once a month. Airborne dust contains "foreign" pollen.

c. Covering and stabilizing samples: At the end of a work period, cover both the samples and the test tubes for holding the stir sticks with a sheet of plastic wrap. Label the test tube rack with the site name, date, your name, and the stage they are in. **SAMPLES MUST BE LEFT WITH ca. 10 CC OF LIQUID ON THEM. A DRIED OUT SAMPLE MEANS YOU MUST START OVER.** If the samples contain water, leave the covered rack on the counter. If the samples, contain acid, label with acid type and leave the covered rack in the hood.

9. HANDLING and PREPARING REAGENTS: Reagents are dispensed from 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. Wash squeeze bottles before refilling; DO NOT just rinse them out. Use distilled or deionized (DI) water because pollen can be present in tap water.

A. Reagents used as supplied by the manufacturer include:

Hydrofluoric acid (HF)	conc. sulfuric acid
glacial acetic acid	TBA - tertiary butyl alcohol
acetic anhydride	100% ethanol

2000cs silicone oil

soda ash (for waste reaction):
technical grade – 100 lb. bags)

B. Reagents needing mixing

Mix by weight-to-volume or volume-to-volume of the chemical *as supplied* and distilled water to obtain desired percentage. Note that the final result is not necessarily a true % of the original. We make 2-3 liters ahead and use over several months. Do all mixing in the hood. Wear gloves, apron, and eye protection.

1. 10% KOH = 100 g potassium hydroxide pellets. plus 900 ml DI water. Accuracy to nearest gram and measure water in a graduated cylinder. Put weighed pellets in container and SLOWLY add water.

2. 10% HCl = 900 ml DI water plus 100 ml conc. hydrochloric acid. Measure with graduated cylinder; pour acid slowly into water.

3. 10% sodium pyrophosphate = 900 ml DI water plus 100 g sodium pyrophosphate. Accuracy to nearest gram and measure water in a graduated cylinder.

4. Acetolysis mixture: Reacts explosively with water. Mix in 48 ml batches in DRY 50 ml graduated cylinder. 9:1 of acetic anhydride and conc. sulfuric acid. Mix just prior to use. Directions in the text.

MATERIALS LIST

Chemicals

See #**9. HANDLING and PREPARING REAGENTS** above.

Equipment

1. C**entrifuge with swinging heads** (fixed heads do not work for pollen processing). Temperature control is not needed and speeds are relatively low, around 3000 rpm.

2. **Centrifuge heads** capable of holding 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. One method is to use a table top centrifuge inside the hood. However, this does expose the centrifuge to the chemical fumes which in turn can shorten its service life. Alternatively, a floor or table-top model outside the hood can have gasket-lined caps that thread onto the centrifuge buckets. The buckets are loaded and capped inside the hood to seal in fumes. We use two IEC floor model Ks. For open air centrifuging, one has an 8 place head (IEC# 253/Fisher 05-423-1) with trunion rings (IEC# 325) and shields (IEC # 320) for 50 ml tubes and with trunion rings (IEC# 310) and 3-place shields (#IEC #303) for 15 ml tubes. For sealed air centrifuging, the other has a 4 place head (IEC #284/Fisher #05-423) that holds capped buckets (IEC # 384S/Fisher #05-334B) suitable for 250 ml bottles. The buckets are fitted with removable adapters that are drilled so that 4 15 ml tubes fit in each bucket. For pollen work, these liners will have to be custom-made of polypropylene or polyethylene because the liners available from the manufacturer are a silica-laced plastic that dissolves and fractures in the presence of HF fumes. We have found that the ones made in our University science shop are no more expensive than the commercial ones. The machinist needs to understand that the four liners must be of equal weight and the drilled holes placed equally because of the need for balance in the centrifuging. Once made, these are essentially indestructible.

3. **Electric hot plate** for maintaining a boiling water bath. Some labs use heat blocks. We use one with the control box is outside the hood.

4. **Vortex mixer** is useful once sample volume is somewhat reduced.

5**. Sonic probe or water bath**: Clay must be removed from samples with much of this done using 7 or 8 µm screens. Screening speeds are significantly improved (from 20+ minutes/sample to 5 minutes/sample using sonic vibration. We are currently using a Crest Ultrasonics Model 2800D acquired by chance when another lab broke up. (1-800-992-7378; www.Crest-ultrasonics.com.) It has a range of settings and is large enough to float 6 to 8 samples. Eric Grimm's lab uses a sonic microprobe.

6. **TBA warming system**: TBA freezes at room temperature frequently making it very 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 temp range block heater (e.g. Fisher cat. # 11-718) without a block. The TBA is dispensed from a plastic dropping bottle, and the bottle sits in a cup set inside the block heater.

7. **Water bath or flat-bottomed saucepan** to be water bath.

8. **Test-tube rack** to fit into pan. SCIENCEWARE/Bel-Art makes round polypropylene test tube racks designed for water baths. (18741 for 15 ml tubes; 18743 for 50 ml tubes)

9. **Acid, base, and solvent storage**: These must be stored separated from each other. It is especially important that the HF be secured and isolated. Squeeze bottles can be kept in carrying trays or plastic basins; stock bottles in appropriate cabinets with spill trays. See the MSDS and your institutions regulations.

10. **Safety clothing**: (a) regular aprons and/or lab coats, goggles and/or safety glasses, and disposable thin gloves as needed/wanted for most of the procedure. (b) for handling HF: use full vinyl coat aprons (much like surgical gowns, they secure in back, extend from neck to below the knees and have full length sleeves – e.g. 8B-23303 or 8B-7600 from Lab Safety Supply 1-800-356-0783), full face shields (e.g. 8B-18116 Lab Safety Supply), and neoprene or nitrile long gloves that extend over the cuffs of the gown. The gowns are ugly, hot and uncomfortable; they are also only needed briefly and are very protective when combined with the gloves and shield.

12. **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 only to plastic for the HF procedures. Use the heavy duty 12 ml tubes (not 15 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 or cracking or splitting. The multiple chemical assaults weaken them over time. Minimum number needed is 16 of each type, but it is helpful to have 30-40 of each on hand.

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

14. **Glassware**:
50 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.

15. **1 adjustable pipette** (if using spike suspension rather than tablets). We have an MLA #1138 that adjusts to between 200 and 1000 µL (Fisher cat. #21-369H).

16. **Pasteur pipettes** (short, disposable) for taking sample check material. Use **small rubber bulbs** to suspend material.

17. **Microscope slides and cover slips** for checking samples.

18. **Sieve systems:** We use 2 "custom" designed systems, one for over 80 µ screen fabrics and another for less than 20 µ fabrics. See Figure 1A and B.

These systems are inexpensive and durable once made. Screen holder sources: Fig 1A is SCIENCEWARE Mini-sieves microsieve set (BelArt #F378450000/Fisher Cat. # 14-306A); Fig. 1B is Tupperware®. For nearest dealer, see the white pages of the phone book. Tupperware® styles change, but any of their large tumbler series or small storage container with a good seal will do. Tupperware® molds its seals to match the cup rim, thus making a secure fit. Any other commercial cup and seal system that is tight would also work.

19. **Screen fabrics**: Our current supplier is SEFAR 1-800-995-0531 Their site is: www.labpak@sefaramerica.com. They sell by yard, but the better buys are their LabPaks which hold several 12" squares and are available in a wide range of nylon or polyester mesh sizes.

20. **Stir sticks**: Glass stir rods or wooden applicator sticks work well. Do not use glass during the HF steps.

21. **Dispensing bottles (500 ml)**: Squeeze bottles are appropriate for most reagents. Also, emptied and cleaned HF bottles are useful for bottles have specially modified anti-drip pour spouts. For the screen steps, spray bottles are very helpful in moving material through the screens.

22. **Marking materials**: lab tape, wax china marker, pens

23. **Vials**: We keep final samples in 1/2 dram shell vials with plastic closures (Fisher 03-339-26A). Do not use vials with shoulders as it makes it difficult to remove material for making counting slides.

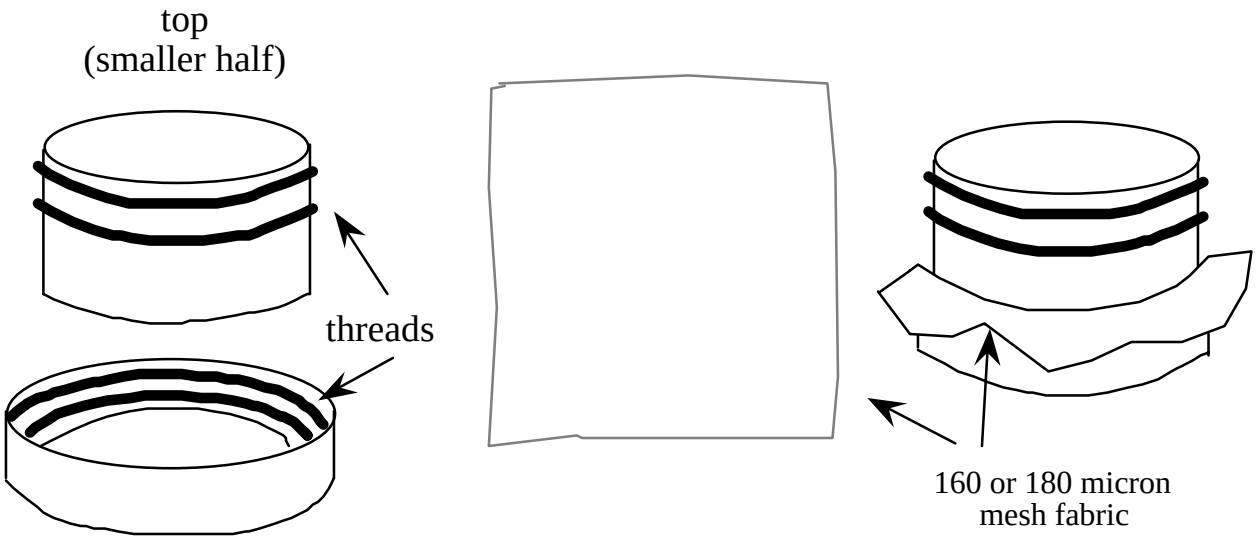
24. **Cleaning supplies**: washbasin, sponges, paper towels, lab wipes, lab detergent, glassware brushes, gloves, etc..

25. **Plastic wrap**: cover samples in when in beakers or tubes between work sessions. A 12’ x 2000’ roll is heavy enough to dispense the wrap without having to pick up the box.

26. **Notebook**: Each preparer should have their own notebook and log the process as it goes. The book should be a permanent bound volume, not just a loose pieces of paper. We also keep a computer log, making two hardcopies of the printout, one filed and the other going to the researcher.

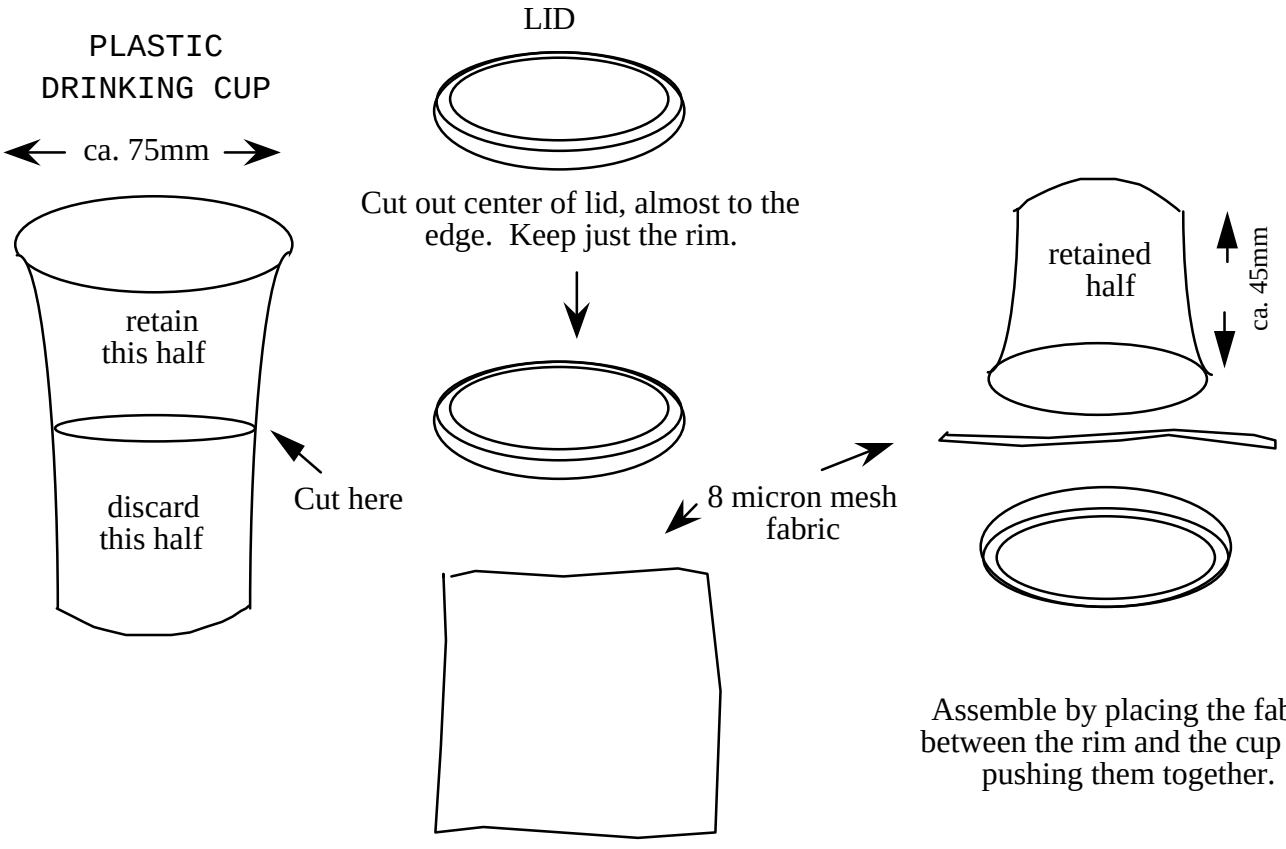
Fig. 1

A. Sieve system for coarse screen fabrics



Assemble by wedging the fabric between the smooth part of the smaller half and the threaded part of the larger half.

B. Sieve system for fine screen fabrics



Assemble by placing the fabric between the rim and the cup and pushing them together.

THE ART OF POLLEN PREPARATION

The purpose of the procedure is to make pollen analysis (microscopic counting of pollen grains) as easy as possible. A sediment residue with a high pollen concentration is generated by using a series of chemical and mechanical treatments to selectively remove as much as possible of the non-pollen components of sediment. 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 10 and 140 µ 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. Hence, the Art of Pollen Preparation.

This procedure takes between 8 and 16 hours to prepare 8 sediment samples for pollen analysis. Except as noted, it can be stopped at the end of each step.

STEP 1: PROCESSING SET-UP

Hint: Start water bath going first - it will be boiling by the time setup is done. Also start spike suspension stirring, if it will be used.

(a) Determine the type and amount of spike to be added. The owner of the samples should provide this information. If the spike is *Eucalyptus* pollen suspension, it will be added now. If the spike is polystyrene microspheres, it will be added at the end of the procedure. For spike preparation and spiking procedures, see separate document. (Spike tablets have a carbonate matrix; if they are used, it is helpful to change the processing sequence to HCl, KOH, Screening.)

(b) Set up a test tube rack with 2 sets of 15 ml test tubes (tt) (polyproylene, graduated, conical) labeled 1-8. One will hold the sample; the other the stir stick for the sample.

Set up your record log in tabular form, including in the heading: date, your name, site name, site location including county, state, country, and researcher name. The table should include columns with: tt# (1-8), core number, depth, sample volume, and spike volume and concentration. Transfer samples to tt, **recording sample details in your notebook immediately after each transfer**. This minimizes mix-ups. Assuming the samples are measured (quantitative), use DI water to rinse 100% of the material into the tt.

(c) Add 2-3 drops of TBA (tertiary butyl alcohol) to wet down any floating particles; centrifuge (see General Information section), and decant.

NOTE ON TBA ADDITIONS: Before every centrifuging (except those after HF, glacial acetic acid, and acetolysis), add a few drops of TBA to the tt. This wets floating particles, making them easier to centrifuge down. Even with this precaution, sometimes sediment particles and pollen remain floating on top of the liquid after centrifuging. Thus, 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 higher setting.

NOTE ON DECANTING: 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 suspension of particles, 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 dumping, you may loose material. DO NOT RE-START A DECANT ONCE YOU STOP IT. Centrifuge and try again. It just takes practice to get the right balance.

NOTE ON STIR STICKS: Stirring sticks can break pollen grains if used roughly. Suspend samples by adding 2-4 ml of liquid, 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 efficiently suspend.

STEP 2: KOH TREATMENT: This step breaks up sediment (as a detergent would) and also removes humic acids.

(a) Add about 6 ml of 10% KOH to each sample and stir gently.

(b) Heat with occasional stirring in actively boiling water bath for 10 to 20 minutes (clays and peat need more; dark lake mud less - consult owner).

(c) Remove from heat; fill with DI water, stir, add TBA, centrifuge and decant.

(d) Rinse with DI water again and check for obvious clumping. Add water to tube, stir, and hold tube up to light. If the sample is very dark to black or full of clumps, repeat a, b, c after centrifuging. In very peaty/organic samples, the KOH step may need 3 or 4 repeats.

(e) Repeat rinsing 3 to 10 times (don't forget TBA). Stop when clear or almost clear. This removes many <3µ particles that interfere with pollen counting. The repeats will vary with the sediment. Record number of rinses.

STEP 3: SCREENING AND "POURING OFF": This step removes large particles and sand. (This can take 1-3 hours).

Set up coarse fabric screen holders (Fig. 1A) with appropriate mesh size. We use 160 µ mesh as a standard. For very high fiber peats you may want to screen twice - once with 180 µ and again with 160 µ to minimize trapping of pollen in the fibers. If you are certain there are no large conifer grains or Onagraceae, you could use 120µ mesh. Set assembled screen on top of a 250 ml beaker and number BOTH beaker and screen holder with the tt number. Transfer the sample onto the screen with DI water and use a spray bottle with DI water to rinse until it appears that everything in washed through excepts large particles and fragments. This usually takes 50-250 ml of water, but can take more.

Next, without contaminating the bottom of the screen holder, check the screen under a dissecting microscope for sediment lumps, larger fossils, sand, insect parts, shells, charcoal, etc. Make very brief notes on what you see. This is helpful to the researcher who is deciding what else to sample for. If sand is present, be especially careful to follow sand removal procedure given next. **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.

Discard material on screens and keep what has gone through.

(b) Concentrate material in the 250 ml beakers by pouring into labeled 50 ml centrifuge tubes using the sand removal procedure below; centrifuge and decant. (Don't forget the TBA.) Repeat until all the material (except sand residue) is in the tube.

SAND REMOVAL: This is very important because larger sand grains are not always fully dissolved by HF (see below). 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. Sand is removed at this step when transferring filtrate from the beaker to a 50 ml tube or the original 15 ml tube depending on how much has to be centrifuged down. Pour the suspension from the beaker *without stirring* into the 50/15 ml tt. When pouring the last approx. 30-50 ml, swirl the beaker gently with a rotating movement and then pour somewhat quickly but without dumping everything. 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.

(c) Rinse material in 50 ml tubes (if used) back into 15 ml tubes using DI water. Again, fine sand can be removed during transfer, using procedures described above. (Don't forget TBA)

STEP 4: ADDITIONAL WATER RINSES: This step removes any remaining organic acids and more fine clay particles. If multiple rinses were done after the KOH step, this step will be minimal.

Rinse samples 2 to 10 times with DI water until supernatant is clear. (Don't forget TBA.) Record number of rinses. NOTE: In high clay samples, it may help to add 5% sodium pyrophosphate, heat 1 minute, and centrifuge. Repeat 2 or 3 times, then rinse with DI water.

STEP 5: HCl TREATMENT: This step removes carbonates.

(a) Add about 1 ml of 10% HCl to each sample initially and stir in very gently, especially if you expect a reaction. Control foaming with a drop or two of TBA. Add about another 5 ml, but do not let reaction overflow the test tube.

(b) Heat samples about 10 minutes in gently boiling water bath. When the reaction is complete, centrifuge and decant into waste container. Often sample material tends to float during this procedure both before and after centrifuging. Be careful that nothing gets discarded. See TBA ADDITIONS note if "floaties" are present after centrifuging.

(c) Repeat if you suspect any carbonates might still be present. This is rare, but you might want to check with the sample owner.

DECISION POINT and SAMPLE CHECK #1 (if needed)

Examine the flow chart. Samples with high silica (sand/silt/clay) content will follow the high-silica path; samples with low silica the high-organics path. If you saw ANY sand during the screening step, follow the high-silica path. If in doubt, follow the high-silica path. Samples that are from very organic (over 50-60% LOI) lakes or extremely high in peat should follow the high-organics path. The key difference here is processing samples so as to eliminate (or minimize) repetitions of the dangerous and time-consuming HF step. This is done by screening samples through a 7 or 8µ sieve to remove as much silica as possible mechanically rather than chemically. On the other hand, the screening may not be necessary in organic-rich samples and can be tedious (10 to 30 minutes per sample) in the absence of a sonic bath or probe to speed

particles through. Examples of high-silica samples are glacial clays, samples from large lakes, samples dating to times of drying conditions, and soils.

If you are still not certain, do sample check # 1. Centrifuge samples, decant, and add about .5 to 1 cc of DI water. Use Pasteur pipette with bulb to suspend the sample, then transfer a drop to a microscope slide. Add coverglass and check at around 250x. If you see crystalline material or a "snow" of 0.5-3 µ 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. Most often you will get a mix.

This description will follow the high-silica path as most samples processed by the LRC need this treatment.

STEP 6: FINE PARTICLE REMOVAL - "NITEXING"

(With high-organic samples, do this step only if determined to be needed after the 2nd sample check.)

Small particles, usually silicates, less than 8µ 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 are digested in the subsequent step with HF. The more efficiently this step is done, the more effective HF is and the more the need to repeat the HF step is reduced.

Set up fine nitex screen fabric sieves (Fig. 1B) with 7 or 8µ fabric. Discard any screens that have irregularities such as staining or holes or stretched weave. Rinse the sample from the test tube onto the screen . This time you are KEEPING the material on the screen. Place each screen either in its own light plastic tub (such as cottage cheese comes in) if you have sonic equipment or on top of a 200 ml beaker if you don't. If you do not have sonic equipment, do in groups of 2 or 3 so unfinished samples are not left in screens until the next day.

Sonic sieving (using an ultrasonic bath): Put about 5 to 10 ml of deionized water in each tub, so some water touches the underside of each screen. Float the plastic tubs in the sonic water bath and turn the sound vibration on. Sample by sample, spray-wash each sample in turn, checking the water in the tub to see if particles are moving through. If the tub water remains clear, then there is little to move through. Remove the sample from the bath. If the water is turning murky, continue spraying. To check if a sample is "clean," discard the water in the tub, repeat the spraying, and check for clarity. We have not done any testing to determine how long grains can be sonicated at what frequencies. Currently we are trying not to exceed 5 minutes, so if samples are high in silicates, sonicate only two or three at a time. The goal is to get rid of most of the ultrafine particles but not all may pass through before you have met the time limit. You can check to see if any pollen has mistakenly passed through by microscopic examination of a bit of the rinsed-through liquid.

Sonic sieving (using an ultrasonic probe): Eric Grimm's lab (Illinois State Museum) uses a low-level hand-held sonic probe which is touched to the screen allowing material to pass through in a matter of seconds (Model TMS130PB Ultra sonic disrupter). Manufacturer is Tekmar-Dohrmann (www.tekmar.com) 4376 Socialville Foster Road, Mason, OH 45040. 800-543-4461-sales)

Hand sieving: 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. In either case, the water passing through the sieve will get all over your hands, so disposable gloves are appropriate.

After screening, rinse the retained material into a clean 250 ml beaker. Then carefully disassemble the screening apparatus and rinse each part into the beaker. The fabric will hold onto the inside surface of the beaker just by putting it there. Transfer the material from the rinsing back into the original test tube, centrifuge (TBA), and decant. Rinse 2 times if pyrophosphate was used.

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.

STEP 7: HF TREATMENT: This step removes silicates (sand, silt, clay). HF dissolves glass. DO NOT USE GLASS RODS OR TEST TUBES.

CAUTION: HF is very poisonous. 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 MSDSs (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 data sheets, 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 two 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 a mixture of soda ash and clay kitty litter to pour on spills - soaks up and neutralizes. If you have an **exposure**, flush the area immediately and continuously with cold water until ready to take the person to an Emergency Room. If you have a **spill**, cover with soda ash/kitty litter mix. Wear full safety garb, place used absorbent in a plastic bag and treat as hazardous waste. If you have a big spill, keep everyone out of the room and call in the Chemical Spill team.

Responsible suppliers (such as Malinkdrodt and Fisher) supply HF in 1 pint (500 ml) bottles with specially designed pour spouts that essentially eliminate dripping. Use acid directly from these bottles. When discarding empty bottles, rinse with a mixture of soda ash and water to eliminate risk to maintenance staff. Keep a few cleaned bottles on hand for dispensing other hazardous reagents.

WEAR LONG PLASTIC APRON WITH FULL SLEEVES SO BOTH ARMS AND LEGS ARE COVERED. USE THE FULL FACE SHIELD AND GLOVES. FEET MUST BE COVERED WITH SHOES (NO OPEN SANDALS).

USED DOMED centrifuge cups to keep fumes out of room air.

Although the HF is dangerous, the risk is entirely manageable if the safety precautions are followed. A good analogy is with driving a car. A car can be very dangerous, but when used properly is a marvelous tool.

The following method reduces risk by minimizing holding of samples in the hands.

(a) 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 (direct from the bottle) to each sample. Avoid any drips. Stir carefully. Transfer rack to actively boiling water bath. Leave the wooden stir sticks in test tubes.

(b) Heat in actively boiling water bath for 20 minutes. Stir 3 or 4 times during the heating. (Leave the room if the hood has poor draw.) When done, remove rack from water bath.

(c) Fill with 95% EtOH (ethanol) to cool (NO TBA); centrifuge 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, then slowly pull the good one out. The broken piece will adhere to the good piece by cohesion.)

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.

Step 8: HOT 10% HCl RINSES: This step is directly related to the HF treatment. It breaks up siliceous colloidal clumps that formed during the silica digestion.

USED DOMED centrifuge cups to keep fumes out of room air for first two rinses.

(a) Add about 6 ml 10% HCl. Stir gently and heat in water bath for about 3 minutes, allowing the sample to get hot. Centrifuge (don't forget TBA) and decant into waste bucket. Watch out for floating particles. See procedure under "TBA Additions" note.

(b) Repeat the hot HCl rinses 2-4 more times. High clay samples usually need 5 total; very organic samples 2 or 3.

(c) Rinse twice with distilled water.

STEP 9: SAMPLE CHECK #2: This is a microscopic check to see how the procedure is going and what if any modifications are needed. If the sample owner can do this check it is helpful, but it is easy to learn what to look for.

(a) Examine the sediment packed in the bottom of the tubes after the last rinse. If you see a small separate gray layer or area, there is still clay in the sample and the HF/HCl steps need to be repeated.

(b) Set up each test tube with about 1-2 cc of DI. First stir each tube, holding the tube to your ear. If you hear conspicuous gritty/grinding sounds, then the HF/HCl steps will need to be repeated. Next set up individual samples on a slide (as in Check #1). Apply a cover slip to each sample. If the cover slip will not lie flat because of grit, then HF/HCl steps needs repeating. Examine under a binocular microscope at around 250x. Look for crystalline materials. If present in sufficient quantity to interfere with pollen counting, then HF/HCl steps need repeating. Also look for large grayish clump. They look a bit like dirty rain clouds. These are colloidal clumps not broken up by the HCl treatment (Step 8). If they are present, then repeating the HF/HCl step is needed, but only 10 minutes of hot HF is sufficient.

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 samples countable.

Heavy liquid separation has been used in some labs in lieu of the HF procedure. We looked at it many years ago, and it is difficult. The original heavy liquid choices were very toxic and repeated extractions were needed. Recently, newer less toxic heavy liquids have been developed. They are extremely expensive and need to be cleaned and reused, making them a possible source of cross contamination. Again, likely several extractions would be needed. It does not appear that any time is saved or that samples would necessarily be cleaner. However, the LRC is still open on this issue and would like to hear of experience in other labs.

STEP 10: ACETOLYSIS: This step removes some organic matter, cleans the surface of the grains, and stains the grains a golden brown.

NOTES: (1) This entire procedure takes about 1 hour and must be done efficiently and with no interruptions.
(2) Water bath needs to be full and ACTIVELY boiling during the actual acetolysis treatment. Don't start until it is.
(3) All reagents are hazardous and the acetolysis mixture reacts explosively with water. Wear apron or lab coat and eye protection. Gloves are suggested. If skin contact occurs, flush area with water.
(4) Be sure waste bucket has additional soda ash. The glacial acetic acid reacts for quite a long time.

USED DOMED centrifuge cups to keep fumes out of room air.

(a) Glacial acetic acid rinses: rinse 2 times with glacial acetic acid, using 5-6 ml/rinse (no TBA). Decant into waste bucket. This removes all water from the samples.

(b) Acetolysis mixture: (Prepare this fresh while the samples are centrifuging after the second glacial acetic acid rinse.)

Prepare a 9:1 acetic anhydride:conc. sulfuric acid mixture in a 50 ml graduated cylinder. Both reagents are hazardous. Keep a supply of each in cleaned and recycled HF bottles for easy handling. Carefully pour 45 ml of acetic anhydride (as it comes from the bottle) into a *dry* 50

ml cylinder. The bottom of the meniscus should be touching the 45 ml line. Add 5 ml of conc. sulfuric acid (as it comes from the bottle) slowly until the bottom of the meniscus touches the 50 ml line. You do not need to stir as the acid sinks into the anydride. The reaction is exothermic, and the cylinder will get quite warm. If you add too much sulfuric acid (over 6 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.

(c) Make sure water bath is full and actively boiling. Go no further until it is right. Acetolysis is only effective near 100°C.

(d) Decant second glacial acetic acid rinse into waste bucket. Stir each sample carefully. Pour about 6 ml of the acetolysis mixture into each test tube (8 tubes x 6 ml = 48 ml). Don't worry about even amounts. Stir samples.

(e) Put samples into actively boiling water bath and heat for **EXACTLY** 2 minutes. Stir samples at least once. (Note: some palynologists use more time for this treatment. We have found it often turns the grains a darker brown, obscuring features. Check with owner, if unsure).

(f) Remove samples from water bath, cool immediately by adding glacial acetic acid (no TBA), centrifuge and decant carefully into waste bucket.

DO (D), (E), AND (F) WITH NO DELAYS. THE SAMPLES MUST NOT BE IN ACETOLYSIS MIXTURE LONGER THAN NECESSARY.

(g) Rinse once with glacial acetic acid.
(h) Rinse 3 times with DI (don't forget TBA).

STEP 11: SAMPLE CHECK 3 (optional): 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. Most often needed is a repeat of the nitex (fine screening) or KOH (excessive clumping).

(a) **Un-clumping:** This is needed if there are a lot of 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.

(c) **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 1-4µ on a side. Nitexing is the removal method of choice, but sometimes not enough crystals pass through. Also the crystals can cluster in large (10-100µ) 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 13: 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 in sequence. DO NOT START UNLESS YOU CAN FINISH THEM BOTH.

(NOTE: If using polystyrene microsphere spike, add at this point and do one more water rinse if the spike is suspended in SeaSpen (see spike preparation procedure). Also increase the centrifuge setting perhaps 10% for the remaining steps. The microspheres have a specific gravity very close to water.)

(a) Rinse once with 10 ml of 95% EtOH
(b) Rinse once with 10 ml of 100% EtOH
(c) Rinse once with 6 ml TBA.
(d) Transfer to properly labeled, dry 1/2 dram shell vials using TBA. When transferring, it is ESSENTIAL that the correct sample goes into the correct vial. Hold the vial up and read it out loud. Hold up the test tube, read the number out loud, and then read the corresponding information in your notebook out loud. This sounds stupid, but it can prevent the most devastating stratigraphic errors.

Centrifuge vials and decant. Vials are readily centrifuged by placing several corks or a piece of dowel into the 15 ml size centrifuge shields to raise the bottom to a good height. On the counter top, vials can be held in drilled wooden blocks.

STEP 14: SILICONE OIL ADDITION: This is the final microscope slide study medium.

(a) Add 3 to 15 drops of silicone oil after determining the correct amount. This is tricky. 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.

(b) Stir samples very thoroughly with a new stirring stick. This is to ensure that all 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 repeating the entire process again.

(c) Cover the open samples in the wooden vial rack by folding a paper towel over everything so the TBA can evaporate (takes 2-3 days). Do not cap them or wrap with plastic wrap. Place in hood. Report to owner that samples are done. If possible, you or the owner should stir them the next day to be sure there is enough silicone oil. The initial presence of the TBA can be distort the estimate of the amount needed.

(d) After evaporation of the TBA, the samples should be checked again for silicone oil volume. Ideally about 1-2 mm of clear oil should sit above the sample. The sample owner is usually responsible for final adjustments. Excess oil can be removed either by allowing settling for 2 or 3 months then carefully pipetting some off (not fully recommended) or 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-suspended after 6 months to 2 years. When stirring a bulky sample after the TBA is gone, the sample should feel only a bit more viscous than the oil alone.

STEP 15: RECORD COMPLETION:

(a) MAKE SURE THAT EVERYTHING IS PROPERLY COMPLETE IN YOUR OWN LAB BOOK. Your book is sometimes checked for details on procedure done to a specific sample, sometimes years later.

(b) TRANSCRIBE A SUMMARY OF YOUR LOG IN THE GENERAL LABORATORY LOG, Currently maintained in a FilemakerPro format. RECORD ANY ERRORS OR DISCARDED SAMPLES. Be certain the sample owner is aware of any problems. Print two copies of the computer document: give one to the sample owner; put the other in the hard copy ring binder. Do not begin another batch until this is done. It is easier to find samples in this log if it is in chronological order.