UCI AMS Facility

Chemical Pretreatment for Bone
Ultrafiltration Method
Dec 26, 2011

Summary

1. Clean, grind, and crush 100- 150 mg of bone per sample, plus standards and blanks (plus small standards and blanks if required)
2. Decalcify 24-36 hrs in 6cc of 0.5N HCl at room temperature in 13mm culture tubes.
3. Prepare and label Centripreps and heavy walled 13mm tubes (weigh tubes).
4. Wash the decalcified bone with MQ water, check pH.
5. (If required) treat sample and a blank with 0.1N NaOH for up to 1 hr at room temp to remove humics, then wash in 0.1N HCl, then MQ.
6. Gelatinize in 5cc of 0.01N HCl overnight at 60°C
7. Pipette samples into ultrafilters, centrifuge 2x, dilute to 5cc with MQ, centrifuge 2x.
8. Prepare the vacuum centrifuge (clean trap, check vacuum, cool down)
9. Pipette samples into heavy wall tubes, freeze down with LN, freeze dry overnight
10. Fill ultrafilters (outside) with MQ, leak check overnight.
11. Weigh heavy wall tubes to determine yields.

I. Introduction to bone treatment:

The bone treatment used at UCI is a modified Longin (1971) collagen extraction followed by ultrafiltration (Brown et al., 1988). The bone is first mechanically cleaned, and then decalcified with relatively strong acid. A weak base treatment may be applied if the presence of contaminating humics is suspected. The resulting crude collagen extract is then hydrolyzed to gelatin at $60^{o}C$ in weak acid, and the gelatin is ultrafiltered to remove small contaminating molecules. The purified gelatin extract is then freeze dried in a vacuum centrifuge.

II. Recording procedures:

The back of the standard UCIG index card should be used for recording details of the bone preparation, in addition to the standard procedures in the front of the card. Some information on the hardness, color, whether it stinks when you grind it (this indicates that organics are present), etc, should be given. If a base extraction for humics is carried out, record that also. Record the weight of bone used and the final yield of ultrafiltered freeze dried collagen extracted.

III. Chemical pretreatment for bone:

A - Cleaning/Grinding: Cleaning includes removal of roots, preservatives or any extra material that may be attached to the sample. The removal of the contaminated bone can be done by washing and/or using the Dremel and dental cutting tools to scrape off the exterior of the bone. If possible, grind off the whole exterior surface of the chunk of bone you want to use. Once it's clean, don't touch it except with gloves (and preferably not at all). Note: The densest part of the bone is best for dating. Don't use spongy bone if you can help it.

B - Crushing: It is best if the size of the bone ranges range from 0.5mm to 2mm. It is easier to work with smaller bone fragments, because demineralization is quicker. However, if fragments are too fine they are more likely to be pipetted away by accident.

1. To crush the bone, first place a large piece of aluminum foil over the work area, then place a metal plate over the aluminum.
2. Cut a second piece of aluminum foil a few square inches and lay it on the plate.
3. If the bone is large and/or hard, dip it in liquid nitrogen to make it more brittle and easier to break. Hold it with pliers and keep it in the liquid nitrogen until all fizzing stops.
4. Place the piece of bone in the middle of the aluminum foil and fold the foil over it.
5. Hammer the bone until it breaks up into coarse powder.

Figure 1: Scraping off the exterior of the bone

Figure 2: Fragments of bone to undergo decalcification

C- Weighing the sample:

1. Place 50 to 150mg of bone chips in a 13mm culture tube labeled with the sample #. Record the bone weight to later calculate the yield.
2. The collagen yield depends strongly on the condition of the bone. In some cases when the bone is very deteriorated, 100mg of bone may not give a useful amount of collagen. In these cases, any radiocarbon date is very likely to be unreliable, because much of the very small amount of organic material left in the bone may be intrusive. Resist the temptation to simply use bigger samples. **If 150mg isn't enough, you probably shouldn't continue**.
3. Prepare bone standards and blanks as well. If the bone preservation looks marginal it's important to have at least one small (say, 25 – 35 mg) background sample and secondary standard in the batch as well as regular sized ones

D-Decalcifying:

1. Add 6cc of 0.5N HCl to the culture tube (fill it almost to the top) and cover with a vented cap.
2. Decalcify for 24 to 36 hours. 6cc of 0.5N HCl should dissolve 150mg of calcium phosphate – $Ca_3(PO_4)_2$ – so you should not have to change the acid.

Note: While this is happening, prepare a set of ultra-filters (see below section E).

3. Check that the bone fragments have stopped bubbling and look translucent.
4. Carefully, pipette the acid out of the culture tube and wash the bone with 4-5 cc of MQ water. Take care not to pipette away any of the bone fragments, but take as much of the liquid as you can. The next step involves 0.01N HCl, and poor pipetting will leave the sample too acidic. Check the pH (blue pH paper) if you're worried.

E- Preparing the ultra-filters: The ultrafilters currently used are Centriprep YM-30 (30,000 molecular weight cutoff). There is a tradeoff between increasing yield and increasing likelihood of contaminant molecules getting through the filter as the cutoff is reduced; and we sometimes use YM-10's (10,000 MW cutoff). Check that you have the right filters: the YM-10's have a green membrane and the YM-30's are clear.

The Centriprep ultra-filter membrane is covered with glycerin, which must be removed before the filter is used. **Once this has been done ALWAYS leave the Centriprep with MQ water in the outer portion.**

1. Fill both parts of the ultra-filter with MQ. Fill the outer part to the fill line and the inner part until close to the top.
2. Fill the sonicator with water to the fill line, place the ultra-filters in the plastic rack, and sonicate for one hour with the heater on.
3. Discard the liquid and wash both parts of the ultra-filter with MQ.
4. Fill the outer part of the ultra-filter to the fill line with MQ water (Figure 3 and 4).
5. If necessary, change the centrifuge buckets to the correct size for the ultra-filters.
6. Centrifuge 3 times for 20 minutes at 3000 RPM. Each time, discard the liquid in the inner and outer part, rinse the membrane and the outer part with MQ, and refill the outer part with MQ. If you have difficulty separating the inner and outer part, label the filter as "tight" and put it to one side (the membrane may tear and you could lose a sample).
7. Fill both parts of the ultra-filter with 0.01N HCl.
8. Sonicate for one hour with the sonicator heater on.
9. Discard the liquid, wash both parts of the ultra-filter with MQ, and leave upright overnight **with the outer part only** filled with MQ.
10. Check the water level in the inner part of the ultrafilter. Any where it's anomalously high are leaky – **DO NOT USE: TRASH IMMEDIATELY.**

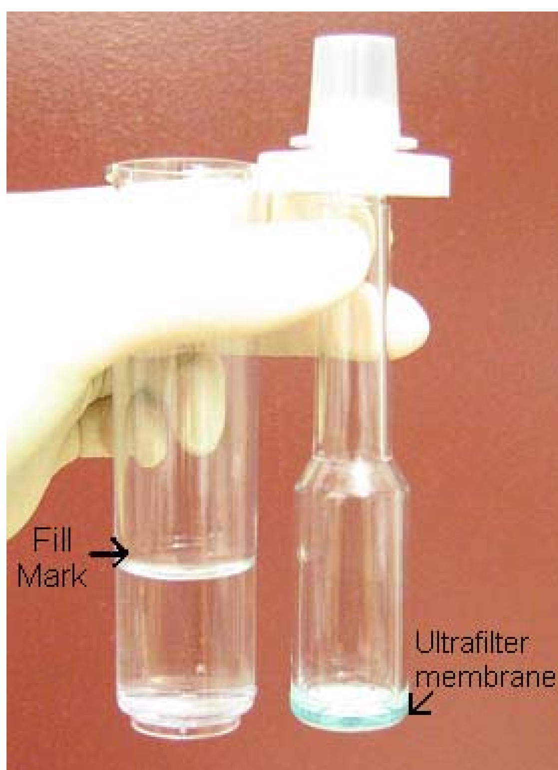

Fig 3: The Centriprep ultra-filter separates into two components. The bottom of the smaller part has the ultra-filter membrane in the bottom of the tube.

Figure 4: Inner part placed into outside part

F- Alkali Treatment (Optional): The treatment with NaOH removes the base-soluble contaminants such as humic acids and some lipids. You may or may not need to add base to your sample: if the decalcified sample is white or clear, skip this procedure. NaOH will eventually dissolve collagen, so don't use it unless you have to. If the sample is dark brown, this may be an indication of the presence of humic contaminants. If that is the case then proceed with the alkali treatment, for the sample and at least one blank:

1. Pipette off the water and add 5cc of 0.1N NaOH (0.1N NOT 1N). Treat for an hour maximum, at room temperature. Check every 15 - 30 minutes. If there is no color in the NaOH, it is not extracting humics - stop.
2. Pipette the base away and wash once with 0.1N HCl and once with MQ water

Note: The NaOH concentration used in the alkali treatment is very important. You may lose your sample if you add the incorrect concentration.

G-Hydrolyzing (Gelatinizing):

1. Pipette off the water and add 5cc of 0.01N HCl (**Note: acid strength is important – check that you have the correct normality).**
2. Set the heating block to 60 °C (about 6 on low scale). Adjust the temperature carefully to within a degree or two. Some of the heating block thermometers are inaccurate when placed directly in the block: if in doubt, put a culture tube 2/3 full of water in the block and immerse the thermometer in it. Collagen is a triple helix and at 60°C the helices break up into individual molecules and go into solution. Overheating will chop up the long-chain collagen molecules, but the collagen is very slow to dissolve below 60 °C. Leave the cultures tubes in the heating block for 8 to 10 hours (eg overnight). Keep the vented caps on the culture tubes.
3. When the collagen is dissolved, remove from the heating block and allow it to cool. Do not heat for more than about 12 hours, as the collagen molecules will start to disintegrate and yields will decrease.

Fig 5: Cold trap and Vacuum Centrifuge.

H- Setting up the freeze dry equipment (at least one hour before use):

1. Empty any liquid out of the glass trap in the refrigerated condensation trap, rinse it twice with methanol, and let it drain very thoroughly on the 2nd rinse. Check that the alcohol level in the metal reservoir is up to the fill line about 1" from the bottom
2. Turn on the roughing pump and check that the vacuum goes to <200 microns on the thermocouple gauge within a few minutes. If it won't go there, check it for leaks and/or call for help.
3. Turn off the pump and vent via the oven (open both oven valves.
4. Turn on the trap and wait at least one hour before using the centrifuge.
5. Turn on the pump again a few minutes before you want to use it.

I- Ultrafiltration:

The liquid to be filtered is placed in the outer part of the ultrafilter, which is spun in a centrifuge. Liquid and small molecules are forced through the filter membrane into the inner part of the filter, while the large molecules of interest remain in the outer section. When the liquid levels in the inner and outer sections are equalized, no more filtration will occur. The liquid in the inner part is discarded and the process is repeated several times to concentrate the large molecules in the smaller and smaller amount of liquid remaining in the outer section.
Note: in the past we've had trouble with filters leaking, and we've saved the filtrate in extra 13mm tubes just in case. This no longer seems to be necessary, but clearly it doesn't hurt…

1. Label a set of thick walled 13mm culture tube with the sample #'s, weigh them, and record the tube weights on the cards.
2. Label a cleaned ultrafilter with the sample #, open it, and discard the water inside. Pipette the dissolved gelatin out of the culture tube, and transfer the liquid to the outer part of the ultra-filter. Pipette VERY carefully to avoid pipetting solids. If there appears to be fine material in suspension, centrifuge the culture tube for 5 minutes before pipetting.
3. Wash the sides of the culture tubes with a few drops of MQ, and pipet into the ultrafilter (avoiding solids).
4. Centrifuge for 20 minutes at 3000 RPM.
5. Open the ultrafilter, wash the outside of the membrane into the outer part of the ultra-filter with a very few drops of MQ (to avoid losing any sample), then pour the contents of the inner part into the regular 13mm culture tube.
6. Repeat steps 3 and 4 again.
7. Dilute what's left in the outer part with MQ to about half way up to the fill line (to reduce the chloride content of what's left)
8. Repeat steps 3 and 4 twice more. This should bring the remaining volume down to about 1cc.
9. Pipette the liquid in the outer part of the ultrafilter into the heavy wall culture tube. Wash down the filter membrane and the sides of the outer part of the ultrafilter with a very few drops of MQ and pipette that also. Avoid pipetting any solid material. The final amount of gelatin solution to undergo the freeze-drying procedure should not exceed about 1cc (about half an inch in the culture tube). If there's too much liquid,

ultrafilter the sample one more time to reduce the volume. Cover the tube with a vented cap.

10. Fill **the outer part only** of the ultrafilters with MQ and leave them upright overnight, to once again check for leaks, then discard.

J-Freezing the samples.

1. Dip the heavy wall tubes two at a time into a dewar filled with liquid nitrogen until the samples freeze. Hold them diagonally and dip them very slowly into the liquid nitrogen so they freeze from the bottom (if they freeze from the top, expansion of the liquid may shatter the end of the tube). Place them in the test tube rack.
2. Repeat until all samples are frozen. This will take a while, so refreeze them all again before loading the centrifuge. Leave the vented caps on the tubes

K-Freeze-drying:

1. While the tubes are still frozen, load them into the centrifuge (with the tubes arranged symmetrically) close the lid, and turn it on. When the rotor is up to speed, the valve connecting the centrifuge to the vacuum system will open automatically. The system should pump to 500-1000 microns on the thermocouple gauge, though this may take several minutes. Pressures may be slightly higher if there are many tubes (10, 20, 30…) in the centrifuge, all contributing water vapor.
2. Leave the samples centrifuging for at least 10 hours. The pressure should go to <200 microns.
3. Turn off the centrifuge. It will isolate itself from the vacuum system and vent to air as it begins to spin down
4. When the centrifuge stops rotating, open the lid and remove samples. They should be white or light tan and should look like fluffy cotton.
5. If you are not going to load new samples to freeze dry, turn off the refrigerated condensation trap. Turn off the roughing pump and open both valves on the vacuum oven to vent the vacuum system.

L- Determining Yields:

1. Reweigh each culture tube (without the caps) and record weights.
2. The difference between the new and original weight as a fraction of the original bone weight gives the ultrafiltered collagen yield. Calculate the yields and record them on the cards. **If a sample gave low yield, check that the ultrafilter didn't spring a leak.**
3. Cap the tubes with gas-tight (non-vented) caps.
4. **Decision time:** if the yield is less than (say) a couple of percent, you will have trouble scraping enough collagen out of the tube to provide aliquots for radiocarbon and for elemental analysis/stable isotopes. You probably shouldn't continue anyway: when yields are that low, much of the very small amount of organic material left in the bone may be intrusive. If it's less than 1 percent, STOP – the material is probably garbage. In addition, if the color of the final product is anything other than white or

light tan, it is a sign that Maillard reactions have taken place, and sugars or other intrusive compounds are covalently bonded to the collagen molecules. **Any radiocarbon date on such material is inherently unreliable…**

Bibliography

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Hedges, R.E.M, and G.J. van Klinken, 1992. A review of current approaches in the pretreatment of bone for radiocarbon dating by AMS. In Long, A., and R.S. Kra (eds), Proceedings of the 14th International 14C Conference. Radiocarbon, 34(3):279-291.

Longin, R. 1971. New method of collagen extraction for radiocarbon dating. Nature 230: 241-242.

Taylor, R.E., 1992. Radiocarbon dating of bone: To collagen and beyond. In Taylor, R.E., A. Long, R.S. Kra (eds) Radiocarbon after four decades: An Interdisciplinary Perspective, pp375-402. New York, Springer-Verlag.

Fisher Part Numbers for Centripreps

YM-10	pack of 8	4321
	pack of 24	4304
	pack of 96	4305
YM-30	pack of 8	4322
	pack of 24	4306
	pack of 96	4307