Isolation of mononucleosomal DNA from Haloferax volcanii

Haloferax volcanii is a type of haloarchaea that enjoys warm temperatures (45 degrees C is optimal) and high (18%) salt concentrations. It is one of the microorganisms that give salt lakes their red color. It also grows much less rapidly than E. coli, and has a generation time of about 3 – 4 hours at 37 degrees. It grows faster at 45 degrees, and can even survive in temperatures up to 60 degrees C.

We chose this organism to study not only because it is pretty and reasonably easy to grow, but also because this variety of haloarchaea has nucleosomes associated with its DNA. It is our purpose to try to isolate the specific regions of DNA that wrap these nucleosomes . Later we will amplify these short (60 bp) regions using PCR, insert them into a vector (pUC19), grown up clones and select for the transformed E. coli that contain the plasmids that carry this nucleosomal DNA code using blue/white screening. These selected colonies will be sent off for analysis of their DNA and a library of mononucleosomal DNA will be created for sequence analysis. We are searching for the code that determines the position of the nucleosomes along the DNA strand.

We grew up a population of Haloferax volcanii (obtained courtesy of (----------))using 18% MGM (see section 2.3 in The Halohandbook), shaking at 200 rpm in a 45 degree incubator. A single colony was cultured in 5 mls of 18% MGM to an OD of about .5. Then this culture was transferred to 100 mls of 18% MGM in a 250 ml flask, and left shaking at 200 rpm, 45 degrees, overnight for about 18 hours. Culture Haloferax volcanii to OD of at least .8 for a large (100 ml) volume. We would like to have 10^8 cells to work with.

Spin down in two 50 ml Falcon tubes at 6350 rpm for 5 minutes. If pellet is reasonably compact and supernatent looks clear, carefully pour off supernatent. If the pellet is very loose and the solution around it still looks cloudy, cells remain in the solution so spin it for another 5 minutes. Wash pellet by adding 10 mls Mnase buffer to tube and placing it in the centrifuge with the pellet on the side opposite to where it was when you took the tube out. That is, if the pellet formed on the outerwall of the tube, position the tube so that the pellet is on the inner wall before closing the lid and spinning. Repeat this wash step one more time for a total of 3 spins, two washes.

Resuspend each pellet in Mnase buffer to a volume of 15 ml, since 25 – 30 mls is the minimum volume that can be processed by the cell homogenizer. Pass mixture through one cycle at a very gentle pressure, less than 10,000. The goal is to open the cells enough to allow the MNase in without disrupting them so much that the MNase has the opportunity to chew up all the DNA it finds. Since these cells lack nuclei, and are experiencing osmotic shock from the change from 18% salt to much much less, they don't need much force applied to them to allow the MNase to enter the cell.
Take 3 mls of cell lysate to digest with MNase. The remainder can be reserved for future testing.

STEP 1: USE MNase TO DIGEST THE DNA THAT IS NOT ASSOCIATED WITH NUCLEOSOMES

Label a series of 2 ml eppendorf tubes with timepoints
(We used: 30", 2.5 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes)

Place each labeled (but still empty) tube in 37 degree water bath.

Place 500 microliters of Stop Solution in a 1.5 ml eppendorf tube and set in the water bath in a convenient place. You will need to remove 50 microliters from it and add it to each tube as their timepoints come up, so be sure this is set up to enhance the accuracy of the test. Have a pipette handy that is set to 50 microliters, and be sure tips are nearby.

Get a timer ready to start counting the seconds up so that you'll know how long you've been timing the tubes. The following has to happen in quick succession so read this over a first so that you know what you need to do and can just zoom smoothly through it:

When everything is set:

- **Add 3 microliters of MNase to the 3 mls of cell lysate**
- **Start the timer while you mix lysate by inverting a few times**
- **Immediately add 500 microliters of cell lysate to each tube.**

About 30 seconds will have passed by now, so:

> **At 30" on the timer, put 50 microliters of Stop Solution into the 30 second timepoint tube. Close the lid on this tube.**

Continue adding 50 microliters of Stop Solution to each tube as their timepoints arrive. Close the lid once the reaction has been stopped so that it will be obvious which tube is to be stopped next.

STEP 2: RELEASE THE NUCLEOSOMES FROM THE UNDIGESTED DNA THAT WRAPS AROUND THEM

- **Add 50 microliters of 5 M NaCl to each tube, close tube, and invert to mix.**

STEP 3: SEPARATE THE NUCLEOSOMES FROM THESE DNA SEQUENCES

- Bring tubes in a rack to the fume hood.
- Add 500 microliters of phenol/chloroform/isoamyl alcohol mixture
- Close carefully and vortex to mix
- Spin in table-top centrifute at 13,200 for 10 minutes

While the tubes are spinning, label fresh 1.5 ml tubes.

At the end of the spin the nucleosomal DNA should be in solution in the top layer of the tube. The phenol/choloroform mixture will be the lower layer, and the nucleosomal proteins (which we can now discard) will appear as a whitish scum on the interface between these layers.

Use a pipette to carefully suck off as much of the top DNA containing liquid layer as possible, being careful not to let the proteins at the interface follow up. Transfer this top layer of liquid to a clean labeled tube.

STEP 4: DIGEST AWAY ANY ASSOCIATED RNA

- Add 3 microliters of RNAse A to each tube.
- Cap and invert a few times to mix.
- Incubate in 37 degree water bath for at least 30 minutes.

STEP 5: REMOVE THE DIGESTED RNA

- Add 500 microliters of chloroform/isoamyl alcohol solution to each tube (also in fume hood, note we do not add phenol this time.)
- Cap, mix, and spin as before, at 13200 for 10 minutes
- While spinning, label clean 1.5 ml eppendorf tubes
- Carefully remove top layer to clean tubes.

Once again, there will be a layer of whitish scum on the interface between the choloroform/isoamyl alcohol and the DNA solution. Be sure not to suck any of this RNA debris up. Also remember to dispose of everything in the hazardous waste containers.

STEP 6: ETHANOL PRECIPITATION OF NUCLEOSOMAL DNA

- **Add 1/7 volume (70 microliters) Na Acetate (3 M, pH 5.3) to each tube**
- **Add 2 to 2.5 volumes (about 1 ml) ice cold 100% ethanol**
- **Mix by inverting**
- **Add 1 microliter of glycogen solution (10mg/ml)**

  The glycogen will be carried down with the DNA and makes a filmy whitish hazy cloud, making it easier to see the DNA precipitate. It is not otherwise necessary, we just add it because it looks interesting. Well, why not?

- **Chill tubes at –80 degrees for thirty minutes.**
- **Spin for 10 minutes at 13200 on table top in 4 degree room.**
- **Carefully pour off supernatant, being sure not to lose DNA pellet.**
- **Add 1 ml 70% ethanol to wash.**
- **Spin for 10 minutes at 13200 (room temp).**
- **Carefully pour off ethanol.**
- **Aspirate away the excess ethanol without touching the pellet.**
- **Resuspend DNA in 30 microliters of TE Buffer that has been warmed to 65 degrees.**

STEP 7: RUN DNA IN 4% NUSEIVE GTG AGAROSE GEL

- Run with 60kb marker
- Use 2 lanes per sample
- Run for a long time at a low voltage to nicely spread out the DNA ladder.
  - 23 volts if overnight
  - 60 volts for 4-5 hours
  - 80 volts for 6 hours