Video Article

DNA Extraction from 0.22 μM Sterivex Filters and Cesium Chloride Density Gradient Centrifugation

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Abstract

This method is used to extract high molecular weight genomic DNA from planktonic biomass concentrated on 0.22 μM Sterivex filters that have been treated with storage/lysis buffer and archived at -80°C, and to purify this DNA using a cesium chloride density gradient. The protocol begins with two one-hour incubation steps to liberate DNA from cells and remove RNA. Next, a series of Phenol:Chloroform and Chloroform extractions are performed followed by centrifugation to remove proteins and cell membrane components, collection of the aqueous DNA extract, and several buffer exchange steps to wash and concentrate the extract. Part Five describes the optional purification via cesium chloride density gradient. It is recommended to work with less than 15 samples at one time to avoid confusion and cut down protocol time. The total time required for this protocol depends on the number of samples to be extracted. For 10-15 samples and assuming the proper centrifugation equipment is available, this entire protocol should take 3 days. Make sure you have the hybridization ovens set to temperature at the outset of the process.

Video Link

The video component of this article can be found at http://www.jove.com/video/1352/

Protocol

Note: *All reagents used in this protocol are aliquoted from lab stocks into smaller working stocks- this is very important to avoid cross contamination of your DNA samples and contamination of lab stocks. Also, when pipetting, make sure to change pipette tips for every addition to avoid cross contamination.*

Part One: Cell Lysis and Digestion

1. Begin this protocol by thawing the Sterivex filters that contain previously concentrated planktonic biomass on ice. For simplicity, here we describe the procedure for processing an individual filter. In practice, we recommend processing 16 or fewer filters at a time.
2. Once the filter has thawed, begin the first of two incubations: one to lyse the cells and remove RNA, and one to break down proteins. For the first incubation, add 100 μl lysozyme (125 mg in 1000 μl TE) and 20 ml RNase A (10 μg/ml) to the filter. Reseal the filter with Parafilm and leave it to rotate in the hybridization oven at 37°C for 1 hour.
3. For the next incubation, add 100 μl Proteinase K and 100 μl 20% SDS to the filter. Reseal using Parafilm and leave it to rotate one to two hours more in the hybridization oven, this time at 55°C.
4. Using a 5cc syringe, transfer the lysate from the Sterivex filter into a 15 ml Falcon tube. Rinse the filter with 1 ml lysis buffer and add the rinse liquid to the lysate in your Falcon tube using the 5cc syringe. Now that you have lysed and digested your cells, you are ready to extract their DNA.

Part Two: DNA Extraction

1. The next step is to use the phenol-chloroform method to extract the DNA from your lysate. Add an equal volume (about 3ml) of Phenol:Chloroform:Isoamyl Alcohol (IAA) to the lysate tube, making sure to change pipette tips for each addition to avoid cross contamination. Vortex for 10 seconds to mix well.
2. Spin the tube at 2500 g for 5 minutes, making sure the centrifuge is balanced. During centrifugation, the phenol-chloroform-lysate mixture should separate into two layers: an organic layer containing the phenol, chloroform, and proteins from your sample, on the bottom, and an aqueous layer, which consists of your DNA, water, and other more hydrophilic molecules, on top.
3. Transfer the aqueous layer (containing your DNA) into a new 15 ml Falcon tube. Be careful not to touch the interface with the pipette tip (always leave a small amount of the aqueous layer behind).
4. To this new tube, add an equal volume (approx 3mL) of Chloroform:IAA, making sure to change pipette tips for each addition to avoid cross contamination. Vortex for 10 seconds. This step helps to remove any remaining phenol from your DNA sample.

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5. Spin at 2500 g for 5 minutes or until aqueous layer is clear. Transfer aqueous layer into a new, labeled Falcon tube and add 1 ml of TE at pH 8.0, being careful not to touch the interface with the pipette tip (always leave a small amount of the aqueous layer behind). This aqueous layer contains your DNA extract.

Part Three: DNA Concentration and Washing

1. The next step is to wash and concentrate the DNA sample in a 15 ml Amicon Ultra centrifuge tube. The Amicon tubes consist of a filter that retains molecules at or above a specified molecular weight (the retentate) and a tube to catch the flow-through (the filtrate). In this case, the filter retains your DNA (the retentate). Transfer your DNA extract from step 2.5 to the filter compartment of an Amicon Ultra tube.
2. Spin at 3500 g for 10 minutes. Check to make sure there is less than 1 ml of liquid retained in the Amicon filter. If more retentate remains, refill the filter with TE and spin again. Make sure to use a fresh pipette tip for every TE addition to avoid cross contamination.
3. Remove the flow-through, or filtrate, to another Falcon tube and save it in the fridge until you have confirmed your final DNA product on a gel (Part Four).
4. Add 2 ml TE buffer to the Amicon filter and spin at 3500 g for 6 minutes, making sure to use a fresh pipette tip for each TE addition to avoid cross contamination. Pool filtrate with that from step 3.3 and save in the fridge.
5. Wash the DNA twice more with TE for a total of three washes (making sure to always use a fresh pipette tip), and saving the filtrate each time (as per steps 3.3 and 3.4). For the last wash, spin until 200 to 500 ml of retentate remains in the Amicon filter. Record the final volume and transfer the retentate (containing your DNA) to a labeled 1.5 ml Eppendorf tube.

Part Four: Determination of DNA Quality

1. Check the DNA quality and estimate the concentration of the DNA by running out your samples on a gel 0.8% agarose gel stained with 1ml ethidium bromide (EtBr) overnight. (Be extremely cautious when using EtBr- it is a known mutagen and suspected carcinogen. Make sure to change gloves often and avoid EtBr transfer to the rest of the lab and to your skin.) Load the samples next to DNA ladders, which serve as size and intensity standards (see next step).
2. In the first several lanes, load ladders with bands of various molecular weights and concentrations. We recommend the following loading pattern: in the first and last lanes (outermost lanes), load 10 μl of 50ng/μl of 1kb+ or 2log ladder. In lanes 2,3 and 4, load 2 μl, 5 μl, and 10 μl, respectively, of 50ng/μl λHindIII ladder. Lastly, load 5 μl per lane of DNA extract with dye for each sample.
3. Run the gel at 15 volts for approximately 16 hours (we recommend running overnight).
4. The next day, photograph the gel using a UV gel documentation system. To determine the molecular weight range of your DNA and its concentration, compare the sample bands to bands of the ladders. Good quality DNA will have high molecular weight (>36kb), and show little evidence of shearing/degradation. See Figure 1.

Part Five: Cesium Chloride Gradient Centrifugation

1. If the DNA quality is good and quantity is sufficient, you can proceed to purify the DNA by performing a cesium chloride (CsCl) gradient centrifugation. Note that this purification step is optional and is not necessary for all downstream applications (for example, if you want to generate fosmid libraries you *SHOULD* purify, whereas if you simply want to perform PCR reactions, purification is not usually necessary). First, label one centrifuge tube for each sample.
2. To each centrifuge tube, add 160 mg of CsCl, 178 μl of genomic DNA. After adding CsCl and DNA to the tube, place a small piece of parafilm on top of the tube and gently invert the tube ten to twenty times in order to mix the components. Do not mix by pipetting, which may cause shearing of the DNA. Next, add 10 μl of 10 μg/μl EtBr to this tube and mix it in the same way. (Be extremely cautious when using EtBr- it is a known mutagen and suspected carcinogen. Make sure to change gloves often and avoid EtBr transfer to the rest of the lab and to your skin.)
3. Make sure that weight of all the tubes are balanced before centrifugation such that the weight differences among the tubes are less than 1 mg.
4. Place the tubes in the rotor using hemostat, close the lid and place the rotor inside the ultracentrifuge.
5. Close the ultracentrifuge door. Vacuum will be applied as soon as you close the door. Run overnight at 100,000 rpm for 18 hours and 20°C.
6. When centrifugation is complete, take out the tubes from the rotor using hemostat and place on a tube rack.
7. Visualize the DNA with blue light transilluminator, or if unavailable, use long wavelength UV light, in a dark room. Wear a pair of amber filter glasses to see the DNA band. See Figure 2.
8. Using a sterile 1cc syringe and needle (26G 5/8), remove DNA band and place it in a 1.5ml Eppendorf tube.
9. To help recover most of the DNA trapped in the dead space in the syringe, rinse syringe with 100 μl TE and add the rinsed solution to the tube. Prepare one tube with 100 μl TE buffer per sample in order to prevent cross contamination.
10. To remove EtBr from the DNA, add an equal volume of water-saturated butanol to the tube, invert the tubes gently ten to twenty times, centrifuge at 10000 rpm for 1 minute and discard the top layer.
11. Repeat washing until the color of butanol is transparent, 3 - 4 more times.
12. Place 4 ml TE in an Amicon Ultra centrifuge tube and add the DNA solution from above.
13. Centrifuge at 3500 g at room temperature, until the DNA volume is reduced to approximately 100-500 μl (approximately 6-7 minutes) and discard the flow-through.
14. Add 2ml TE to Amicon filter and centrifuge at 3500 g for 6 minutes, making sure to use a fresh pipette tip for each addition to avoid cross contamination. Repeat twice.
15. Concentrate to a final volume of 50-100 μl by additional centrifugation as necessary and transfer the DNA solution on the filter to a new 1.5 ml Eppendorf tube.
16. Add 40 μl TE to the Amicon filter and pipette up and down along both filter membranes to wash out any remaining DNA. Add this solution to the tube in 5.15.
17. Place a Microcon YM-30 filter unit into a Microcon tube and prewash the Microcon by adding 200 μl autoclaved water and centrifuging at 10000 rpm for 7 minutes.

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18. Add the DNA solution from 5.16 to the prewashed Microcon in order to further concentrate the DNA. Centrifuge at 5000-10000 g for 1 - 3 minutes. Check the amount of liquid on the filter and repeat centrifugation until the amount of liquid on the filter is reduced to approximately 50 µl.
19. Place the filter unit upside down in a new Microcon tube and centrifuge at 1000 g for 3 minutes. The ideal amount of concentrated solution is 50-60 µl.
20. Measure and record the concentration of the DNA on a Nanodrop and check peak quality (should be 260nm).
21. Transfer a small aliquot of the DNA to a clean eppendorf tube for working stock and freeze at minus 20. Transfer the rest of the DNA to a second clean eppendorf and freeze at minus 80.

Representative Results:

When this protocol is done correctly, you should see a gel image after step 4.4 in Determination of DNA quality similar to Figure 1. Actual DNA concentration of extracts will vary depending on the source of the sample. After step 5.7 in CsCl gradient centrifugation, genomic DNA illuminated by blue light should look similar to Figure 2.

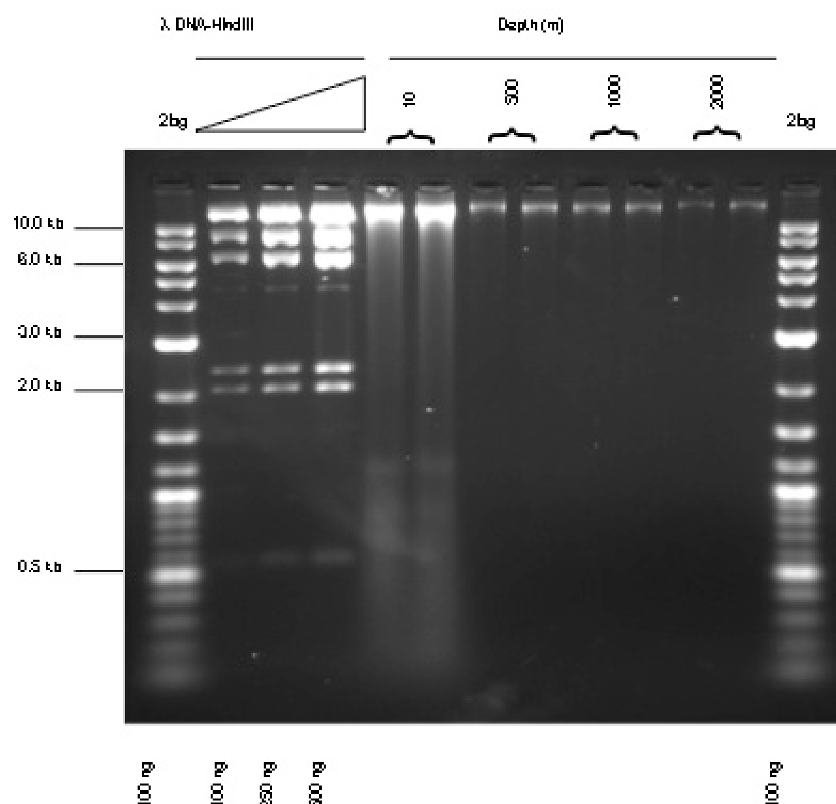

Figure 1. 0.8% agarose gel electrophoresis image of high molecular weight DNA collected from four depths in the subarctic Pacific Ocean (in duplicate), stained with the intercalating agent ethidium bromide (10 mg/ml). Gel was run at 15V for ~16hrs in 1X TBE gel running buffer. Sample bands are of good quality showing little evidence of mechanical shearing (shows single bands or smears as opposed to multiple bands) although the 10m extracts retain some RNA carry over (see smear in the 0.5 to 2.0 Kb range).

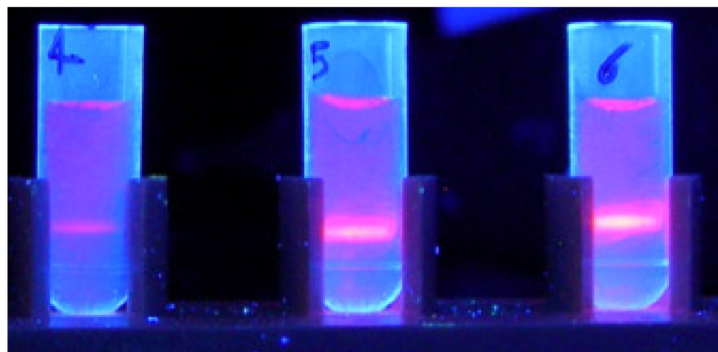

Figure 2. Genomic DNA band illuminated by blue light after CsCl gradient centrifugation.

Discussion

Depending on how many samples are to be processed, this can be a time-intensive procedure due to the two one-hour incubation steps, and the repeated washes and centrifugation steps. It is best to plan two whole days for this procedure to leave plenty of time. If there are problems getting the final extract volume in the Amicon tube to reduce down to 200-500μl during DNA concentration and washing, try doing additional TE washes and centrifugations (repeat Part Three) until the appropriate volume is leftover. Also, if the extract smells acutely of chloroform, it is best to do additional washes until the smell lessens to make sure all of the chloroform is removed. Again, all reagents used in this protocol are aliquoted from lab stocks into smaller working stocks- this is very important to avoid cross contamination of your DNA samples and contamination of lab stocks. Also, when pipetting, make sure to change pipette tips for every addition to avoid cross contamination.

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