

Experiment 6

Large-Scale Purification of Plasmids pRIT4501 and pRIT4502 by Cesium Chloride Density Gradient Centrifugation

Now that you have identified your two recombinant plasmids, you need to produce large-scale preparations of each so that you can study them further. To do this, you will prepare lysates of 500 ml cultures and purify the DNA by isopycnic sedimentation. Although you could have used the alkaline lysis procedure from Experiment 5, you will use a different method here. In the current procedure, 500 ml of cells are concentrated and spheroplasted. They are then treated with Triton X-100, a very mild detergent, which produces holes in the cell wall without actually destroying it. The bulk of the plasmid will leak out of the cell wall but the chromosome remains trapped within and is removed when the cells are pelleted.

The resulting plasmid solution is purified further by density gradient centrifugation. CsCl and ethidium bromide are added to the lysate and transferred to ultracentrifuge tubes. The tubes are spun at 40,000 rpm for two days. During this time, the CsCl forms a gradient and the molecules migrate according to their density until they float at their individual isopycnic points (the point in the gradient that equals the buoyant density of the molecule). However, plasmid DNA and contaminating chromosome have about the same density and cannot be separated easily. This is rectified, however, by the addition of ethidium bromide.

Density is a function of AT/GC ratio, but it is also a function of conformation. For supercoiled DNA, there is more DNA per unit volume than for relaxed DNA. Intercalation of ethidium bromide into DNA causes the helix to unwind (negative supercoiling) and become more relaxed. However, negative supercoiling only relaxes the DNA to a point. Further unwinding induces supercoiling in the opposite direction. When the DNA is circular and the ends are connected, the plasmid “kinks up” into a very tight knot. Thus, ethidium bromide causes the plasmid density to be increased. This principle can be demonstrated by the famous “rubber band experiment”.

The Famous Rubber Band Experiment

1. Obtain two identical rubber bands. Cut one and leave the other intact.
2. Holding one end of the cut rubber band, twist the other end 20 times. Hold on to both ends but let the rubber band relax and note the extent of collapse.
3. Holding the uncut rubber band in the middle, twist it 20 times at a point on the opposite side of the circle. Hold on to both points but let the rubber band relax and compare the extent of collapse in cut and uncut rubber bands.
4. Tape both twisted rubber bands into your lab notebook.

The Day Before

1. Prepare two 500 ml flasks of LB broth in liter flasks and autoclave. When cool, add enough ampicillin to give a final concentration of 50 $\mu\text{g/ml}$.
2. Inoculate each flask with one of your transformants and incubate over night shaking at 37 $^{\circ}$.

**Cell Lysis
First Day**

1. Harvest your cultures as follows:

Transfer half of each culture to a 250 ml polypropylene bottle, balance, and centrifuge at 7,000 rpm for 10 minutes in a Sorvall GS-A rotor. Decant the supernatant.

Pour the remaining half of the culture into the bottle now containing only the pellet. Centrifuge again and decant. ***Be very careful not to mix your cultures!!***
2. Resuspend the combined pellets in 15 ml LB broth and transfer to a 50 ml polypropylene centrifuge tube. Centrifuge the cells at 10,000 rpm in a Sorvall SS-34 rotor. Decant and freeze the dry pellet until next lab.

In a normal lab situation you would continue through to the end of the protocol. However, due to the time constraints of our class schedule, we will stop here and continue next period. Some lysis protocols include a freeze/thaw step to break down the cell walls. Although this protocol does not, a freeze/thaw cycle can only help.

**Materials for
Second Day**

25% sucrose
lysozyme solution (5 mg/ml)
0.25 M EDTA, pH 8
Triton X-100 lytic mix

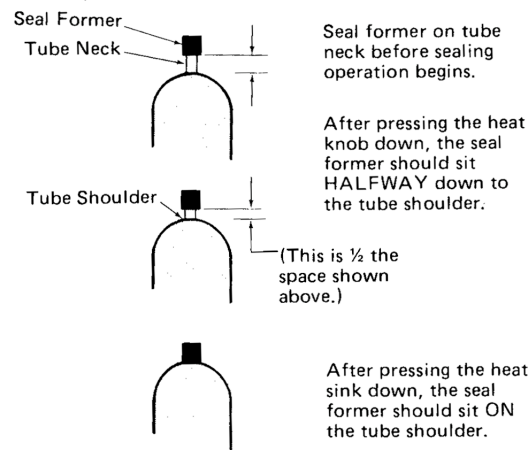
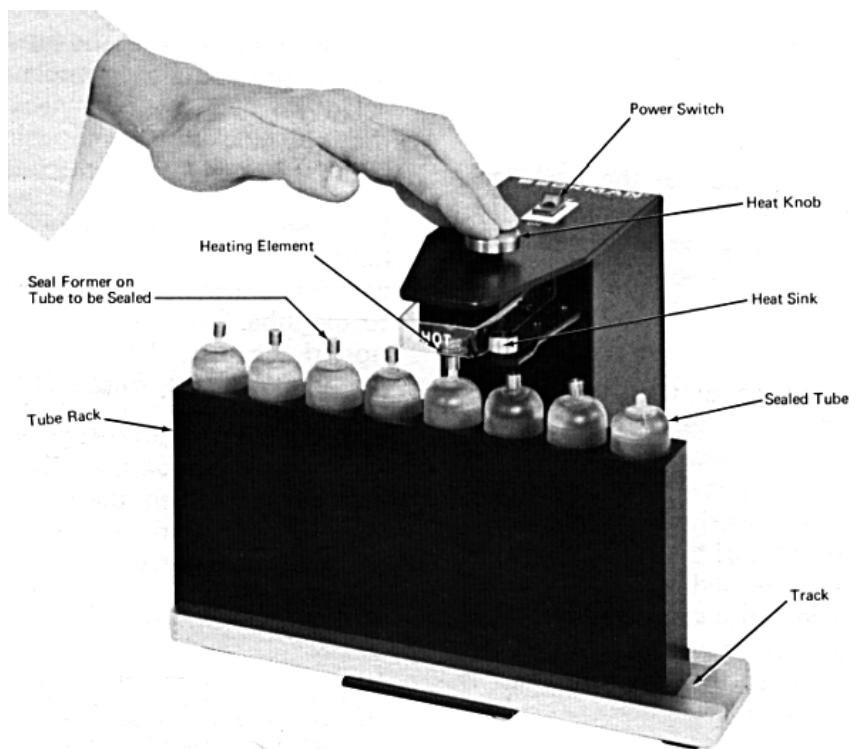
**Cell Lysis
Second Day**

1. Prepare an ice bath and chill all of the materials.
2. Thaw the cell pellets and resuspend each in 1 ml ice-cold 25% sucrose. Place the tubes on ice for 30 minutes.
3. Add 0.2 ml lysozyme solution, mix, and place on ice for 10 minute. Lysozyme breaks down the glycosydic bonds in the polysaccharide backbone of peptidoglycan. The isotonic environment created by the sucrose prevents the spheroplasts from lysing.
4. Add 0.4 ml EDTA, mix, and place on ice for 10 minutes. EDTA is a chelating agent that absorbs divalent cations (Ca^{++} and Mg^{++}), which are required by most bacterial nucleases. When the cells lyse, these nucleases will no longer be regulated and will begin degrading plasmid DNA. The addition of EDTA therefore inactivates these nasty enzymes.

5. Add 1.6 ml Triton X-100 lytic mix. Mix by rocking the tubes *very gently* and place on ice for 20 minutes. Triton X-100, which is a detergent, gently lyses the cells by dissolving the lipids in the cell membrane. Because of the gentle lysis, most of the chromosomal DNA remains unfragmented and becomes entangled in the cellular debris. Plasmids, being considerably smaller, diffuse out into the surrounding solution. Your samples should become very viscous and “snotty.”
6. Centrifuge the lysate at 17,000 rpm for 30 minutes. The resulting supernatant contains approximately 95% of the plasmid in about 3.2 ml. This may be purified further by CsCl density gradient centrifugation.

Preparing the Gradient

1. Transfer your lysate to a plastic centrifuge disposable tube. Add 2 ml TES and 4.9 g CsCl. Shake gently to dissolve. CsCl becomes very cold as it dissolves.
2. Obtain a Beckman “Quick-Seal” tube and a long-tip Pasteur pipette. Carefully transfer your DNA to the “Quick-Seal” tube by inserting the tip of the pipette into the tube’s neck.
3. Using a narrow gel-loading pipette tip, carefully add 200 μ l of ethidium bromide (10 mg/ml) to the sample so that the ethidium bromide floats on top. Visible light will cause nicks in DNA when ethidium bromide is present. It is not necessary to work in the dark, but on the other hand, you shouldn’t go out of your way to expose the DNA to bright light.
4. Fill the remaining space in the “Quick-Seal” tube up to, but not into, the neck with mineral oil. If you do not fill up the unused space, the tube will collapse during the centrifugation.
5. Balance the tubes. The class will gather together and balance all of the tubes at once. You may remove liquid by wrapping a kim-wipe around the mouth of the tube and squeezing. If extra weight is needed, add additional mineral oil.
6. Seal the tubes according to the diagrams below:



7. Rock the tubes to mix the ethidium bromide and place them in a Beckman Ti50 ultracentrifuge rotor and spin at 40,000 rpm for at least 44 hours at 15°. During most of the spin, the CsCl will sort itself out into a density gradient -dilute at the top and concentrated at the bottom. Towards the end of the spin the samples will begin to sort themselves out according to buoyant density.

Collecting the Gradient

1. At the end of the run, carefully remove your samples and look at them under blacklight. You should see two bands. The upper, narrower band is residual chromosome that was not removed during lysis. The lower, heavy band is plasmid. You should also see a pellet of RNA on the bottom and a cap of residual protein floating on top.

- Carefully clamp the tube to a ring stand. Puncture the top of the tube with a wide-bore hypodermic needle (16 g x 1 1/2") attached to a 10 ml syringe. *Never use a wide-bore needle to collect a gradient. It will create currents in the tube that will mix up the gradient.* The entry point should be just below the plasmid band. The needle should be pointed down so as to not disturb the plasmid, and away from the pellet so as not to become contaminated with RNA.

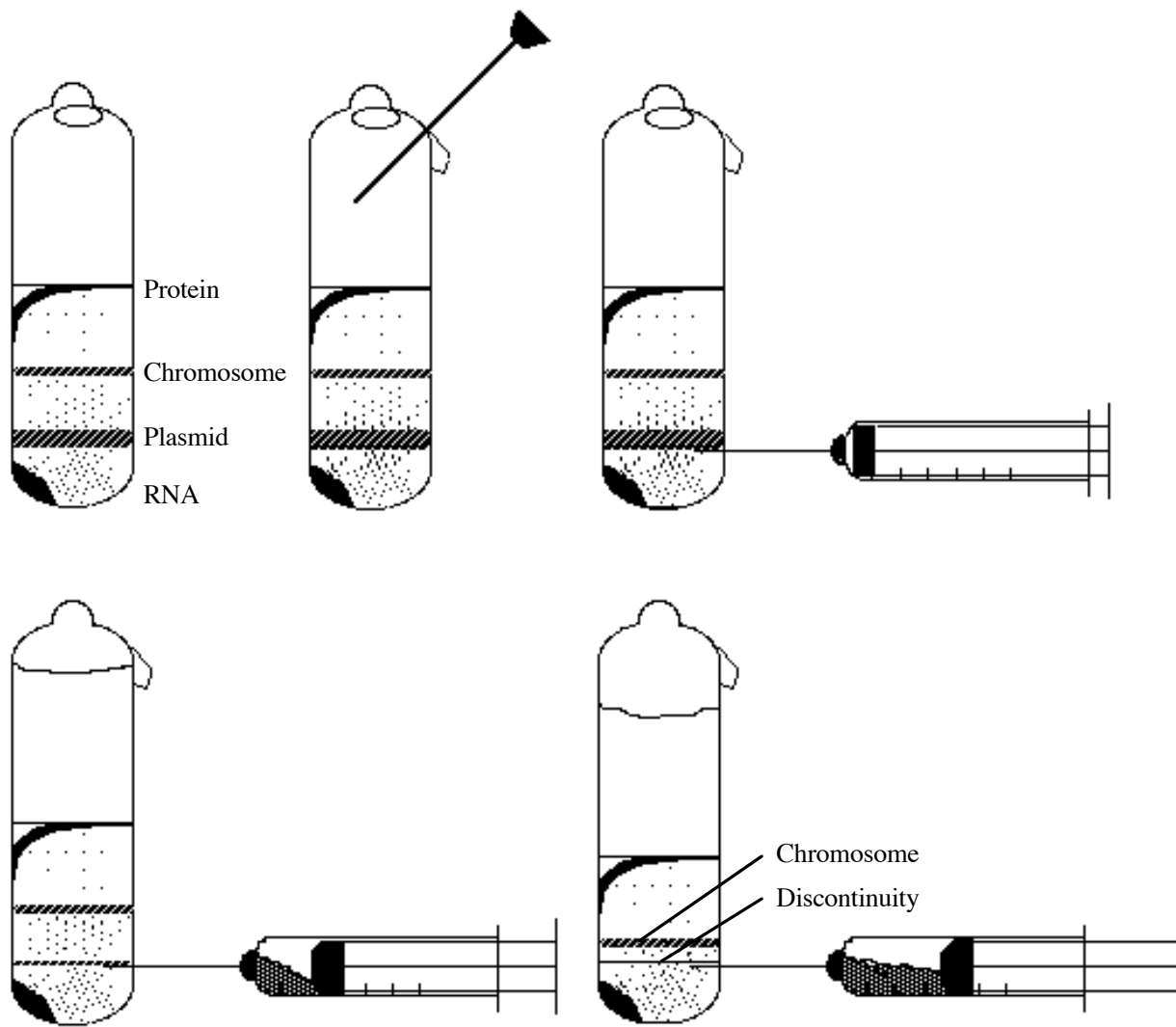
This can be a very stressful operation the first time you try it. The most common error is to jerk your hand back when the needle suddenly goes through the wall of the tube. This results in pulling the needle out, and the plasmid running out through the hole. It is a rare person who can stick the needle back in the hole in time to save the DNA.

The second most common error is to push so hard that the needle penetrates the wall, goes through the gradient, and comes out the other side. Your other hand will be holding the tube to steady it, so you must be mindful of where you put your fingers.

- When the needle is in the gradient, turn it so that the beveled side is up and tip it so that it touches the underside of the band. Carefully draw off the plasmid.

As you draw off the plasmid, you are introducing a discontinuity in an originally continuous gradient. There is a great enough difference in refractive index on either side of the discontinuity to see an interface. This interface is in the same position as the plasmid band and it could fool you into thinking that there is still plasmid there. It is good to get all of the plasmid, but if you get too greedy, you will end up collecting chromosome DNA that is following just behind the plasmid band.

- When you have recovered the plasmid band, withdraw the needle and allow the gradient to dribble into a beaker. Cut the tube in half so that the remaining ethidium bromide and mineral oil can drain into the beaker for disposal by the instructor.



6. Extract the ethidium bromide with isopropanol saturated with cesium chloride. Draw up a large volume of isopropanol into the syringe, rock it a few times, and hold the syringe needle-up for a moment to let the phases separate. The DNA will be on the bottom. Near the collection setup there will be a flask containing a 25 ml pipette with a rubber bulb. Holding the syringe needle-up, insert the needle into the bulb and eject the isopropanol into the bulb, where it will run down the pipette and collect in the flask. Repeat as many times as necessary to remove all of the ethidium bromide, but do at least two extractions.

7. Obtain a dialysis bag and two dialysis clips. Drain as much liquid out of the bag as possible and then clip one end. Carefully transfer the DNA into the bag. Half close the clip on the top of the bag and pull the neck of the bag through the clip until all of the liquid is forced into the bottom. Snap the clip shut. The bag should be taut. Dialyze the DNA over night in 4 liters of TE buffer.
8. On the following day, carefully remove the DNA with a Pasteur pipette and store it in a microfuge tube for use in Experiment 7.

TES Buffer

1 M Tris, pH 8.....	2.5 ml
0.25 M EDTA, pH 8.....	1.0 ml
5 M NaCl.....	0.5 ml
water.....	46.0 ml

25% Sucrose

1 M Tris, pH 8	2.5 ml
0.25 M EDTA, pH 8.....	0.2 ml
sucrose.....	12.5 g
water.....	47.3 ml

Triton X-100 Lytic Mix

10% Triton X-100.....	1 ml
1 M Tris, pH 8.....	5 ml
0.25 M EDTA, pH 8.....	25 ml
water.....	69 ml

Isopropanol Saturated with Cesium Chloride

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Prepare a solution of CsCl (1 g/ml) in water. Add enough to saturate 250 ml of isopropanol. Once isopropanol is saturated, it becomes immiscible with water. You will know when the isopropanol is saturated because the excess CsCl will form a clearly visible layer at the bottom of the bottle. If you use unsaturated isopropanol to extract ethidium bromide from your plasmid prep, the isopropanol will draw water from your sample and the CsCl will precipitate.

Preparation of Dialysis Tubing

1. Cut the tubing into lengths of 15 - 20 cm.
2. Boil for 10 minutes in a large volume of 1 mM EDTA, pH 8, and 2% sodium bicarbonate.
3. Rinse well in distilled water.
4. Boil for 10 minutes in distilled water.
5. Allow to cool, and store in water in refrigerator. Make sure that tubing is *always* wet.
6. *Always handle tubing with gloves!!*