Separation of DNA from Vector Sequences by Sucrose Gradient

One of the most important parameters determining the success of transgenic experiments is the quality of the injection DNA. The DNA needs to be of the highest purity and completely free from vector sequences. The following method will yield such a DNA preparation and is the approved methodology for the Transgenic Mouse Core Facility.

Note: Every precaution should be taken that the DNA does not become contaminated. This is one of the major causes for lack of transgenic animals. Do not use powdered gloves. Sterility of reagents and supplies is very important. Filter sterilize all reagents using a 0.2 :m filter.

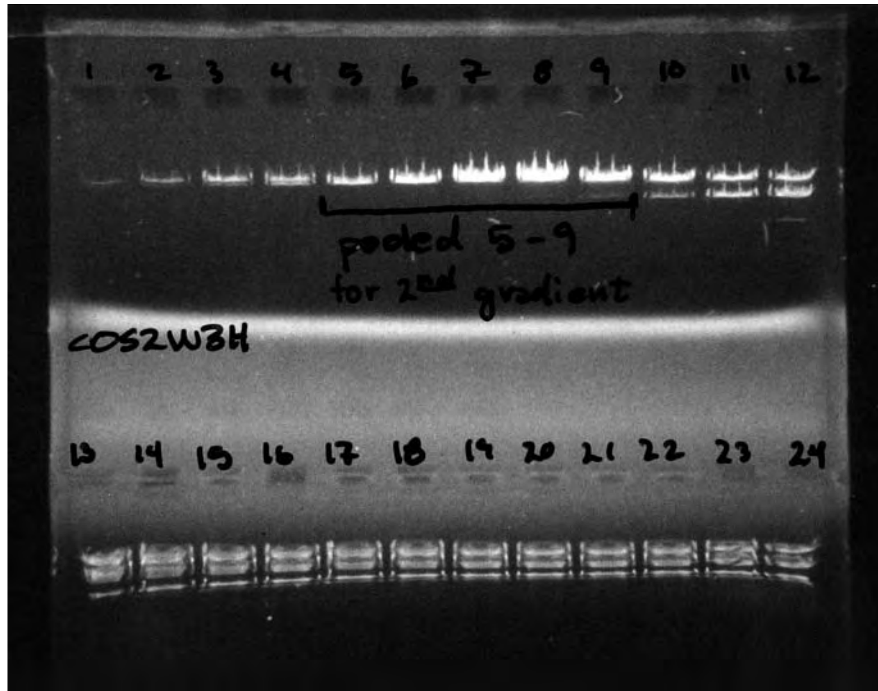
1. Perform a preparative restriction enzyme digest with 50-110 :g (amount depends on the size of the construct of cesium chloride gradient purified or Qiagen-purified vector containing the DNA of interest. The idea is to obtain approximately 15-40 :g of purified insert. The total volume of the digest should be 100-200 :l. The choice of restriction enzymes is specific to the individual vector and insert.
2. During the restriction digest incubation, prepare the linear 10%-40% sucrose gradient that will be used to separate the vector from the insert. **See Linear Sucrose Gradient Preparation at the end of this outline.**
3. At approximately two hours, check the digest for completeness by running 1-2 :l of the reaction on an agarose gel. The gel should be slightly overloaded to effectively observe any low level of incomplete digestion. Photograph the gel for review. If necessary, spike the digestion mixture with additional restriction enzyme.
4. Once the digest is determined to be complete, inactivate the restriction enzymes for 15 min at 65-70EC.
5. Carefully layer up to 100 :l of the inactivated preparative digest on the top of one sucrose gradient.
6. Centrifugation conditions depend on the insert size. For 15-20 kb inserts, centrifuge at 32,000 rpm in a SW41 rotor for 20-24 hours at 20EC. For 5-10 kb inserts, centrifuge at 35,000 rpm in a SW41 rotor overnight at 20EC. Set the conditions for centrifugation so that there is a slow acceleration rate and no braking during deceleration. The following formula is used for calculating any change in speed or time:

$$T_1(RPM_1)^2 = T_2(RPM_2)^2$$

7. Remove 1.5 ml from the top of the gradient. Carefully insert a rubber or cork stopper that already has two 22-25 gauge needles inserted all the way through the stopper. Attach a 5 ml syringe to each needle. This device allows one to control the flow of fractions.
8. Carefully place the tube with the stopper in a column clamp attached to a stand.
9. Organize 30 to 40 1.5 ml microcentrifuge tubes in a rack under the tube.
10. Pierce the bottom of the tube with a sterile 22 gauge needle.
11. Collect 5 drop fractions. Use the syringes attached at the top for flow control.
12. Analyze the fractions on agarose gel at the appropriate percentage to resolve vector from insert. Load 15 :l per lane. No loading dye is necessary due to the

sucrose content. **Store the fractions at 4EC** until the gel is finished running. Photograph the gel for review.

EXAMPLE:



13. If the vector is not completely separated from the insert DNA, which is often the case, then select the fractions that are approximately 90% rich with respect to the insert. Ethanol precipitate the DNA from the fractions by adding 1/10 volume of 3M sodium acetate pH 6.0 and 2.5 volumes of 100% ethanol to each individual 1.5 ml tube. Place the tubes at –80EC for 15-30 min. Centrifuge at 14,000 rpm in a microcentrifuge at room temperature for 15 min. The pellet will mostly likely be small to invisible. Wash once with 70% ethanol. Resuspend each pellet in 20 :l TE. Make sure that each pellet is in solution and then combine into one tube.
14. Prepare a second set of gradients.
15. Follow steps 5-12.
16. If the separation appears complete after the second gradient, immediately transfer the fractions on ice to the Transgenic Mouse Core Facility personnel or store at **4EC for no more than 72 hours** (essentially over a weekend). Include a photograph of the gel for determination of which fractions to pool for dialysis.

Linear Sucrose Gradient Preparation

The gradient maker used for this procedure should be thoroughly cleaned and be free of any contaminants. Rinse each chamber of the gradient maker three times with double distilled water and then three times with sterile double distilled water.

If only one DNA is to be purified prepare a balance tube. Thus, the following procedure describes the conditions for preparing two gradients.

1. Prepare 100 ml of a 10% sucrose solution and a 40% sucrose solution

10% Sucrose

10 g	Sucrose
5 ml	1M Tris-HCl pH 8.0
20 ml	5M NaCl
2 ml	0.5M EDTA

40% Sucrose

40 g	Sucrose
5 ml	1M Tris-HCl pH 8.0
20 ml	5M NaCl
2 ml	0.5M EDTA

Adjust to 100 ml in a volumetric flask using sterile double distilled water. Filter using a 0.2 :m filter.

2. 12 ml of each solution is loaded into the gradient maker in order to prepare 2 gradients. The design of each gradient maker will determine the specifics of where to load the solutions, stirring rate, etc. Pump rate should be slow at ~ 1 revolution of the pump/20 sec. A small stiff metal tube is connected to the end of the pump tubing and placed in the tube in such a way to allow the gradient to form from the bottom of the tube. So the 10% solution is pumped in first and the 40% is last. **DO NOT ALLOW AIR BUBBLES TO PUMP INTO THE TUBE AS THIS MEANS THE END OF THE GRADIENT!!!** Carefully remove the metal tube from the centrifuge tube so as not to disturb the gradient.