TRANSGENIC TECHNOLOGY CENTER

PREPARATION OF TARGETING VECTOR DNA FOR ELECTROPORATION

TTC

3-15-12

1. Isolate the targeting vector plasmid DNA by cesium chloride density gradient purification. A second choice for purification is Qiagen columns (#12143 or # 2362). Be aware that the DNA isolated using a number of other commercial columns can be toxic to ES cells. Therefore we only recommend these two methods. The second column purifies

2. Linearize 300 µg (yes 300 µg!) of purified plasmid DNA with the appropriate enzyme in a volume of 300 µl. Choose an enzyme that cuts once or twice within the vector back bone only and try to leave ~ 200 bp of vector DNA on the end of the short arm. After incubating the preparation for the appropriate amount of time, analyze 1 µl of the reaction by agarose gel electrophoresis to ensure the DNA is linearized. If it is not, continue the digest. If the DNA preparation has a significant smear of ethidium stained material above the linearized vector, it is likely contaminating bacterial DNA from the plasmid prep. – consider re-preparing DNA.

3. Extract the DNA preparation with an equal volume of phenol chloroform/isoamyl alcohol (25:24:1) – Mix the contents of the tube by gently shaking, not vortexing when isolating DNAs of moderate size i.e. 10-30 kb.

4. Centrifuge the tube for 15-20 seconds at room temperature. If the organic and aqueous phases are not well separated, centrifuge for a longer time or at a higher speed.

5. Transfer 90% of the upper, aqueous phase to a fresh tube and repeat the process.

6. Add an equal volume of chloroform: isoamyl alcohol to the tube, shake gently and centrifuge for 15 minutes at room temperature.

7. Take 90% of the aqueous phase from the tube and transfer to another tube. Precipitate the DNA by adding ammonium acetate and ethanol.

8. For ammonium acetate precipitation, estimate the volume of DNA solution and add 0.3 volumes of ammonium acetate (7.5M). Mix well. Add 2 volumes of ice cold ethanol. Mix well and chill to -20°C for at least 1 hour.

9. Centrifuge at 0°C for 10 minutes at 1200 g and decant alcohol.

10. Wash the DNA pellet with ice-cold clean 70% ethanol. Add ice-cold 70% ethanol gently until the tube is 2/3 full. Vortex briefly and centrifuge at 0°C for 10 minutes at 1200 g. After the 70% ethanol wash, the pellet does not adhere tightly to the wall of the tube so be careful when pipetting off the supernatant. Repeat the procedure **2 times** . This part is critical.

11. After the third wash, remove as much fluid as possible by inverting the tube and pulling off adherent fluid with a capillary tube. Let the pellet air dry for a brief period and resuspend the pellet in 50 μl of sterile, nuclease free H_20. Use 2 μl of DNA stock to check the O.D. 260 value to determine the concentration and the O.D. 260/280 value to check DNA purity. Dilute the stock to a concentration between 1 to 2 μg/μl.

12. Label the tube appropriately with tape not just writing on the tube, and store the sample at -20°C until delivery to the Center.

13. Fill out the appropriate form for requesting gene targeting and provide the sample to the Core facility. The Core staff will re-check the concentration by fluorimetry and assess the quality of the preparation by gel electrophoresis.

Notes:

1. It is widely believed that the routine phenol-chloroform step of the DNA purification is one of the most crucial steps in the overall success of the targeting project.

2. Sterility is an important issue, therefore, especially after extractions, manipulations should be made in a tissue culture hood with sterile reagents, tubes, etc.

3. Linearized DNA is much more efficient for gene targeting so take the requisite time to verify the complete digestion before proceeding to clean up the DNA.

4. Linearized DNA is more susceptible to exonuclease degradation, therefore once suspended, the DNA should be stored at -20°C.