LIGATION AND TRANSFORMATION

INTRODUCTION:

Two crucial procedures in cloning are the ligation of the foreign DNAs to the vector DNA and the transformation of bacteria using those ligated DNA constructs (the recombinant molecules). Ligation is accomplished using the enzyme DNA ligase (usually from the bacteriophage T4). It requires ATP and magnesium ions to catalyze the reaction of a 3'-OH and a 5'-P on double-stranded DNA to form a phosphodiester bond. The DNA ends can be cohesive ends, such as those formed between molecules that have been digested with the same restriction endonuclease, or they can be blunt ends. Ligation between cohesive-ended molecules is much more efficient than ligation between blunt-ended molecules. Because of this, when ligating blunt-ended molecules, the DNA and ligase concentrations must be higher than when ligating cohesive-ended molecules.

Several impurities can cause inhibition of the enzyme. One of these is ADP, which is formed from ATP during the ligation reaction. Normally, ligase comes from the manufacturer with the reaction buffer that includes ATP, but if it does not, a good grade of ATP should be used, since lower grades of ATP often contain significant quantities of ADP. Contaminants from fragments eluted from agarose gels can often inhibit ligase (and other enzymes, as well). Use of spermidine (at 3-5 mM) can alleviate some of this inhibition. However, when eluting DNA from gels for use with enzymes an agarose that is made specifically for use with enzymes should be used. Monovalent cations (e.g., Na^+ and K^+) at moderate concentrations (100-200 mM) can also inhibit ligation. However, when used in conjunction with polyethylene glycol (PEG) they can actually increase ligation. Many different molecular weights of PEG can be used, but the most effective concentrations are between 5 and 15%. PEG alone can also increase ligation somewhat, although the addition of monovalent cations greatly increases its effects. For an unknown reason, however, while ligation appears to increase for PEG concentrations from 10-15%, the transformation efficiencies for molecules thus ligated decreases. Therefore, a PEG concentration of 5% (with PEG, average molecular weights 3,000 to 8,000) is generally used. At this concentration, ligation and transformation are both increased significantly.

Transformation of the bacteria begins with making the bacterial cells "competent," which simply means that they are made permeable to the foreign DNA. This is normally accomplished by the treatment of cells in the mid-log phase of growth with calcium chloride. It is thought that this pokes holes in the bacterial cell wall (but I haven't seen any direct evidence of this). By this method, from $10^5$to 10^7 transformed cells can be obtained from a microgram of introduced DNA. In 1983, Hanahan (J Mol. Biol. 166:557-580) carried out a detailed analysis of the conditions for optimizing transformation of bacteria. He was able to increase the transformation efficiencies up to 10^8 to 10^9 transformants per microgram of introduced DNA. He estimated this as being equivalent to one in every 400 DNA molecules being effectively taken up by the cells. His final conclusions stated that a solution containing magnesium ions, calcium ions, and rubidium and/or potassium ions, as well as dimethyl sulfoxide (DMSO), dithiothreitol (DTT) and hexamine cobalt (III) ions yielded the best results.

We will be using T4 ligase, in conjunction with 5% PEG and 100 mM NaCl, to ligate the foreign DNA into the plasmid. After ligation, competent cells will be made according to one version of the Hanahan method (described above). The ligated DNA will then be used to transform the

competent bacteria, which will then be selected for by plating on medium with antibiotics. Further selection will then be done by transferring the resulting colonies onto two different antibiotic plates to screen for the recombinant clones.

STEPS IN THE PROCEDURE:

A. Making Competent Cells:

1. Spread some E. coli cells onto an LB (agar) plate and grow the bacteria overnight at 37 °C.

2. Remove one colony and suspend it in 25 ml of SOB medium in a sterile 250 ml flask.

4. Incubate this at 37 °C, with shaking at 200 rpm. While this is incubating, set up the ligation reactions (part B, below).

5. After about 1 hour, and for each subsequent half hour, remove 2 ml of this solution into a Falcon 2054 tube and assay for culture growth in the Spec 20 @ 550 nm.

To use the Spec 20:

a. Turn on the machine by turning the left-hand dial. Wait for a few minutes for the lamp to warm up.

b. Turn the same dial (and no test tube in the machine) so that the meter shows 0% transmittance (infinite absorbance).

c. Next, place a tube filled with 2 ml of LB medium (without bacteria) into the machine and set the right-hand dial so that the meter shows 100% transmittance (0 absorbance).

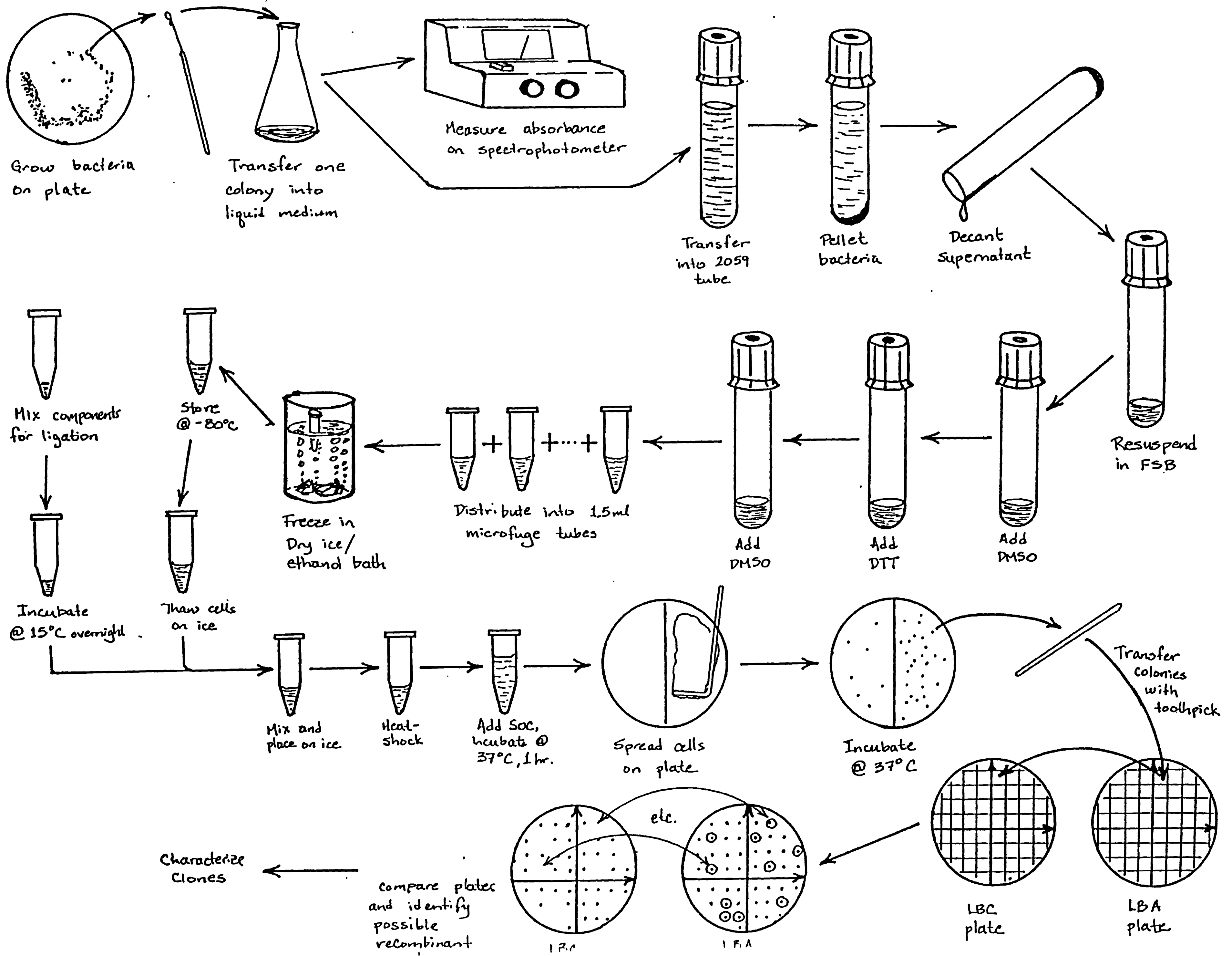
d. The machine is now set to read the densities of the bacterial cultures. If any of the dials are subsequently moved, reset the 0 and 100% values before reading the next samples.

6. When the optical density (O.D.) has reached around 0.45-0.55 (which corresponds to around 5×10^7 cells, indicating the culture is in the mid-log phase of growth), transfer 10 ml of the culture into a Falcon 2059 tube and centrifuge @ 2500 rpm for 10 minutes in the cold.

7. Resuspend the pellet in 2.5 ml of FSB and place on ice for 5 minutes.

8. Add 70 μl of DMSO and leave on ice for 5 minutes.

9. Add 100 μl of 0.5 M DTT and place on ice for 10 minutes.


Grow bacteria on plate
Transfer one colony into liquid medium
Measure absorbance on spectrophotometer
Transfer into 2059 tube
Pellet bacteria
Decant Supernatant
Resuspend in FSB
Add DMSO
Add DTT
Add DMSO
Distribute into 1.5ml microfuge tubes
Freeze in Dry ice/ ethanol bath
Store @ -80°C
Mix components for ligation
Incubate @ 15°C overnight
Thaw cells on ice
Mix and place on ice
Heat-shock
Add Soc, incubate @ 37°C, 1 hr.
Spread cells on plate
Incubate @ 37°C
Transfer colonies with toothpick
LBC plate
LBA plate
etc.
compare plates and identify possible recombinant
Characterize Clones

10. Add 70 µl of DMSO and place on ice for 5 minutes.

11. Divide into 200 µl aliquots and flash-freeze in a dry ice/ethanol bath, then place into a -70 °C freezer until needed.

B. Ligation:

1. Set up the ligation reactions as follows:

50 ng of EcoRI-cut vector DNA
0.25 - 1 µg of *Eco*RI-cut insert DNA
2 µl 1M NaCl
2 µl 50% PEG 3350
2 µl 10X ligation buffer
sterile H_2O to bring volume to 19.5 µl,
then add 0.5 µl T4 DNA ligase

[Note: Most companies supply a buffer that includes the PEG and NaCl. Check the buffer prior to adding these two components. Adjust volumes appropriately.]

2. Mix completely, but try to avoid bubbles in the mixture. Place at 15 °C overnight.

3. When ligation is complete, remove a 2 µl aliquot, mix it with 1 µl of RS, and run it on a gel when you have the opportunity, to assay for ligation. Place the samples (with and without RS) at -20 °C until needed.

C. Bacterial Transformation:

1. Thaw out 200 µl of cells on ice for each transformation (for a ligation reaction) or use 20 µl when transforming using an uncut vector (control experiment).

2. Add the ligation reaction mixture (containing the ligated DNA molecules, from part B, above) to the competent bacterial cells and swirl to mix the DNA and cells. Place the tube on ice for 30 minutes.

3. Place the tube at 42 °C for 60 to 90 seconds to heat shock the bacteria, then place on ice for 1-2 minutes.

4. Add 800 µl of SOC medium to the tubes and place at 37 °C, with shaking, for 1 hour.

5. Spread 200 µl of this onto LBA (agar) or X-gal/LBA plates and incubate overnight at 37 °C.

D. Selection of Recombinant Colonies:

-- pBR325 transformations --

1. Place an LBA and an LBC plate each over the grid provided on the following page (that will later be used to determine the locations of the transferred colonies). Be sure to make a mark on the plates so that the orientation of the plates over the grids can be determined later.

2. Using one end of a sterile toothpick, carefully stab one of the colonies from the plate containing the transformed bacteria (from part C, above).

3. Next, stab it into a single grid location on the LBA plate, then onto the same grid location on the LBC plate. It must be in this order, LBA then LBC, because the recombinant pBR325 molecules will have lost their chloramphenicol resistance, and you do not want to carry over any of the chloramphenicol to the ampicillin plate.

4. Do this either until you have run out of room on the plate, or until you have run out of colonies to transfer.

5. Incubate both plates at 37 °C overnight and record the results. Cells containing religated pBR325 will grow on both plates, while those that have foreign DNA inserted in the chloramphenicol gene of pBR325 will grow only on the ampicillin. These are the colonies we will be interested in when we will be extracting the plasmid DNAs from the bacteria and performing some characterization of the recombinant molecules.

-- Transformations with α-complementation --

1. Place a new X-gal/LBA plate on one of the grids on the following page. Stab a single white colony (which may be a recombinant) with a sterile toothpick and transfer the colony to a simgle grid square on the new plate. Do this until you have selected all of the white colonies or until no grid squares remain. You should also transfer one or more blue colonies also, so that you know that the color selection is active on the new plate.

2. Incubate both plates at 37 °C overnight and record the results. These are the colonies we will be interested in when we will be extracting the plasmid DNAs from the bacteria and performing some characterization of the recombinant molecules.

E. Long Term Storage of Bacterial Cultures:

1. Bacterial cultures generally die if stored at 4°C for more than a week in liquid or agar. Freezing at -20 °C or -80°C may also kill many cultures. However, a liquid culture with glycerol (to a final concentration of 15 - 50 %) is made, the culture can be frozen and kept indefinitely. First, grow a culture in 2059 Falcon tubes or in 1.5 ml microfuge tubes at 37°C with shaking (150 - 200 RPM) overnight.

2. Dispense into 1.5 ml microfuge tubes (sterilized) and add the sterilized glycerol to

each. Any volume can be used, for example 800 μl of culture plus 200 μl of glycerol.

3. Label tubes clearly. Freeze at -80°C. Rapid freezing by immersion into a dry ice/ethanol bath increases survival rates of the bacteria.

4. To revive them, a sterile loop or toothpick can be scraped across the top of the frozen culture, and then spread on an appropriate medium at the appropriate temperature.

SOLUTIONS:

SOB medium

2% tryptone
0.5% yeast extract
10 mM NaCl
2 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
(adjust pH to 6.8 to 7.0)

SOC medium

SOB with 20 mM glucose added

FSB medium

10 mM potassium acetate (pH 7.0)
100 mM KCl
45 mM $MnCl_2$
10 mM $CaCl_2$
3 mM hexamine cobalt chloride
10% glycerol
(adjusted pH to 6.4)

DMSO (dimethyl sulfoxide, molecular biology grade)

0.5 M DTT (dithiothreitol)