

Protocol

Part 1: Ligation Reactions

For your ligation, you should use 50 to 100 ng of the prepared backbone. The volume needed for this amount can be estimated by comparing the intensity of the purified backbone to the 3 Kb marker, which will have 125 ng of DNA. Once you've decided on the volume of backbone to use, choose a volume of prepared PCR product that will give a 1:4 ratio of backbone to insert. You should also set-up two control ligations. The "bkb+insert, no ligase" reaction controls for any errant uncut plasmid that might have wandered into your solutions. The "bkb only, plus ligase" reaction controls for religation of the backbone, from any singly cut plasmids for example.

The contents of each ligation will be

	bkb + insert, no ligase	bkb only, plus ligase	bkb + insert, plus ligase
pCX-NNX bkb	? μ l	? μ l	? μ l
PCR insert	? μ l	xxx	? μ l
10X Ligation Buffer[^]	1.5 μ l	1.5 μ l	1.5 μ l
T4 DNA Ligase	xxx	0.5 μ l	0.5 μ l
Water	To 15 μ l not including volume of enzyme		

[^]New England Biolabs sells 10X Ligation buffer to use with their ligase. It contains ATP so must be kept on ice.

1. Assemble the reactions in eppendorf tubes but not in the order listed. Please ask if you are unsure what order to assemble the components.
2. When the ligation mixes are complete, flick the tubes to mix the contents, quick spin them in the microfuge to bring down any droplets, then incubate the reactions at room temperature for at least 10 minutes.

Part 2: Precipitation of DNA

In this step, salts and buffers are removed from the reactions. DNA is precipitated with salt and ethanol. Yeast tRNA is added to the precipitation as "carrier," allowing you to better visualize the DNA pellets. The salts are washed from the pellets with 70% ethanol. The tRNA is not removed. Rather it enters the bacteria with the ligation DNA, but is then rapidly degraded.

1. Add 20 μ l 3M sodium acetate to each tube.
2. Add 5 μ l tRNA to each tube.
3. Add 200 μ l cold 100% ethanol to each tube and vortex.

4. Spin in a room temperature microfuge 15 minutes. Be sure to orient your tubes in the microfuge so you know where your pellets should be and balance your tubes with those of another group or with a water-filled eppendorf tube.
5. When the spin is done, locate the pellets in each of the eppendorf tubes. They may appear as solid white dots at the bottom corner of the tube or they may appear to be a diffuse white smear along the wall of the tube. Both are OK. Carefully remove the ethanol from the pellets with your P1000, taking care not to disturb the pellet. You do not have to remove every last drop.
6. Wash the pellets with 500 μ l cold 70% ethanol. This is done by dribbling the 70% ethanol along the wall of the eppendorf tube that is opposite your pellet and then removing the 70% ethanol with the same pipet tip. Again you should not disturb the pellet and you do not have to remove every drop of liquid in the tube. If the pellet seems to float away from the wall of the tube, you can re-spin the tubes for 2 minutes with the liquid to adhere the pellets to the wall again.
7. Once you have washed all your pellets, give the tubes a quick spin in the microfuge to bring down any droplets of ethanol that cling to the sides of the tube then remove any remaining liquid from the tubes using your P200. Allow your tubes to dry in the hood for 10 minutes. All the ethanol must be removed or evaporated.
8. Resuspend the pellets in 15 μ l sterile water. This is done by adding water to the tubes and mixing. If the DNA does not readily go into solution, it helps to heat the DNA in the 42°C heat block, then vortex and pipet up and down several times. Bring any droplets down to the bottom of the tubes with a quick spin in the microfuge.

Part 3: Bacterial Transformations

You will perform 4 bacterial transformations, one for each of the ligation mixtures as well as one transformation with 5 ng of plasmid DNA to assess transformation frequency.

1. Prewarm and dry five LB+AMP plates by placing them in the 37°C incubator, media side up with the lids ajar.
2. Get an aliquot of competent cells from one of the teaching faculty. Keep these cells on ice at all times. There should be at least 200 μ l of cells in each tube. Aliquot 50 μ l of cells into 4 clean eppendorf tubes.
3. Add DNA to each tube of cells as shown in the table below.
4. Flick to mix the contents and leave the tubes on ice for at least 5 minutes.
5. Heat shock the cells at 42°C for 90 seconds exactly. Use your timer.
6. Move the samples to a rack on your bench then use your P1000 to add 0.5 ml of LB media to each eppendorf tube. Invert each tube to mix.
7. Plate 200 μ l of each transformation mix on LB+AMP plates, plating the bkb+insert transformation twice. Note: After passing the glass spreader through the alcohol burner and letting the ethanol burn off, the spreader may still be very hot, and it is advisable to tap it gently on a portion of the agar plate without cells in order to equilibrate it with the agar (and if it sizzles, it's way too hot). Once the plates are done, wrap them with colored tape and incubate them in the 37°C

incubator overnight. One of the teaching faculty will remove them from the incubator and set up liquid cultures for you to use next time.

Tube	Transformation	Add
1	positive control plasmid	1 μ l (5 ng) of pCX-EGFP
2	bkb + insert, no ligase	5 μ l
3	bkb only, plus ligase	5 μ l
4	bkb+insert, plus ligase	5 μ l