

Yeast-Mediated Ligation Protocol adapted from a Dartmouth lab protocol by Erik Andersen

A. Construct design (see Figure 1 to help clarify this description)

1. Make a large stock of pRS426 cut with BamHI and XhoI. We perform the restriction reaction for at least 12 hours to ensure that much of the pRS426 plasmid is cut. Store the vector at a final concentration of 100 ng/ μ L – no clean-up is required. Every yeast-mediated ligation should include a no insert control to determine how much uncut or re-ligated pRS426 is in the transformation.
2. Design primers with at least 50 bp of overlap between the fragments to be ligated. Make PCR amplicons of 1-2 kb. The outer most fragments should have homology (from the primer) to the pRS426 plasmid. This protocol can be used to ligate DNA fragments to make rescuing, reporter (GFP, RFP, etc.) or tagged constructs.

Here is the primer design:

5F: GTAACGCCAGGGTTTTCCCAGTCACGACG...add your 20-mer here
3R: GCGGATAACAATTTACACAGGAAACAGC...add your 20-mer here

B. PCR amplification of yeast-mediated ligation target

1. To reduce the number of PCR-induced mutations, use Platinum HiFi Taq from Invitrogen in as few cycles as possible. HiFi Taq can reliably amplify large (15 kb) fragments. It is best to determine the smallest cycle number to produce a robust band.
2. Amplify the 1-2 kb fragments. You don't need to clean these reactions prior to addition to the yeast transformation mix.

C. Yeast transformation using cut pRS426 vector and PCR fragments

1. Grow up 10 mL of FY2 yeast in YPD at 30°C overnight.
2. In the morning, check the O.D. after diluting a small amount of overnight culture to 1:10 or 1:20 of the original concentration. O.D.s above 0.3 are usually not within the spec's linear range.
3. Dilute your overnight culture to an O.D. of 0.2 in 50 mL of YPD. Use the following equation to determine the volume of the overnight culture to add to fresh YPD to get the desired O.D. in whatever time you want:

$$\text{Volume of overnight culture to use} = \frac{((\text{Final culture volume} \times \text{O.D. desired}) / 2^n)}{\text{O.D. of overnight culture}}$$

n = (expected # of generations – 1) Remember yeast generation time is 90 min.

Let's say that we want a 50 mL final culture volume with an O.D. of 0.8 after three hours (two generations) and our starting O.D. was four.

Volume of overnight culture to use = $((50 \times 0.8)/2^1)/4 = 5$ mL, so we would inoculate 45 mL of YPD with 5 mL of overnight culture.

4. Spin cells down in a clinical centrifuge at top speed for two minutes.
5. Rinse cells in 25 mL of sterile water and spin again at top speed for two minutes.
6. Decant water and resuspend cells in 1 mL of 100 mM lithium acetate
7. Transfer to a microfuge tube and spin at top speed for 15 seconds.
8. Resuspend cells in 400 μ L of 100 mM lithium acetate, if the O.D. = 1. If it was less, adjust the volume.
9. Keep on the bench at room temperature until ready to use.
10. Vortex cells to mix.
11. Pipette 50 μ L of cell mixture to each empty transformation microfuge tube.
12. Spin for 15 seconds and pipette off supernatant.
13. To the cell pellet, add the transformation mix components in the order listed below.
14. Add 2 μ L of each fragment and 100 ng of cut vector (1 μ L) to the yeast transformation. Then add water to bring the total volume of what you added to the cells to 360 μ L.
15. Vortex each tube to resuspend each pellet and mix reagents
16. Incubate at 30°C for 30 minutes.
17. Mix by inverting, then heat shock in a water bath for 30 minutes at 42°C.
18. Spin cells down for 15 seconds at full speed.
19. Pipette off transformation mix.
20. Rinse cells in 1 mL sterile water by gentle pipetting, spin at full speed for 15 seconds.
21. Decant most water until 100-200 μ L remain; resuspend gently.
22. Plate out all of the cells on Ura- plates.
23. Grow for 2-3 days at 30°C.

D. Yeast “smash-and-grab” DNA preparation*

1. Scrape off as many colonies as possible using a glass slide or a plastic pipette tip. Deposit the cells into 500 μ L of sterile water.
2. Spin the cells down at full speed for 15 seconds to pellet and remove the supernatant.
3. Add 200 μ L of lysis buffer, 200 μ L of phenol/chloroform solution and 300 g of 0.45-0.5 mm glass beads. Beads can cause tubes not to close completely, so be careful.
4. Vortex for 1-2 minutes (remember to wear gloves).
5. Spin for 10 minutes at full speed; remove 100 μ L of aqueous supernatant to a fresh tube.
6. Add sodium acetate pH 5.2 to 0.3 M and 2.5 volumes of ethanol.
7. Spin for 5 minutes at full speed; discard the supernatant; rinse pellet with 70% ethanol; spin again to remove excess ethanol.
8. Air dry pellet for two minutes and then resuspend in 50 μ L of TE.

*Alternatively, you could score the yeast colonies by colony PCR and then only prepare DNA from a correct yeast clone.

E. Electroporation of shuttle vector with inserted fragments

1. Add one μL of DNA from previous step to 25 μL of GeneHog Electrocompetent cells in the electroporation cuvette (BioRad Gene Pulser Cuvette cat. #165-2086) on ice.
2. Dry the cuvette and put into the electroporator. We use a BioRad Gene Pulser II.
3. Set the resistance to 200 ohms, the capacitance to 25 μF and the volts to 2.5 kV for a 0.2 cm cuvette with 25 μL .
4. Press the pulse buttons and release after the beep.
5. Put the cells on ice.
6. Add 200 μL of SOC medium and recover for one hour at 37°C.
7. Plate out the cells on an LB-Amp plate and grow overnight at 37°C

F. Selection and verification of correct constructs

1. Pick as many colonies as you want to score by colony PCR or culture PCR for correct constructs. For large ligations (>20 kb), we routinely score 25-50 colonies by growing each in a deep-well 96-well plate and then performing PCR on one μL of each culture. Sometimes the correct clones grow more slowly or differently than others on the plates. Pick a variety of colonies to score.
2. Prepare several independent clones and sequence important junctions and regions to make sure the construct is wild-type.

Reagents

yeast shuttle vector pRS426 – containing ampR and URA3 genes

BamHI and XhoI for cutting pRS426

Gene-specific primers

HiFi Taq polymerase from Invitrogen and PCR reagents

FY2 yeast strain

YPD plates and media for growth of FY2

Minimal media plates without uracil for selection of transformants

50% polyethylene glycol 3350

1 M Lithium Acetate

salmon sperm DNA, resuspended and boiled for 10 minutes (VWR cat. #62111-454)

“smash-and-grab” lysis buffer

0.5 mm glass beads

phenol/chloroform

Sodium acetate pH 5.2, 100% and 70% ethanol, TE

electroporator (BioRad Gene Pulser II)

OneShot Gene Hog Electrocompetent cells from Invitrogen (cat. #C808003),

SOC growth medium

Transformation mix, per sample

240 μL 50% PEG 3350 (w/v)

36 μL 1 M Lithium Acetate

50 μL carrier DNA

34 – X μL sterile water

X μL total volume of cut plasmid and PCR fragments

“Smash-and-grab” lysis buffer

2% Triton X-100

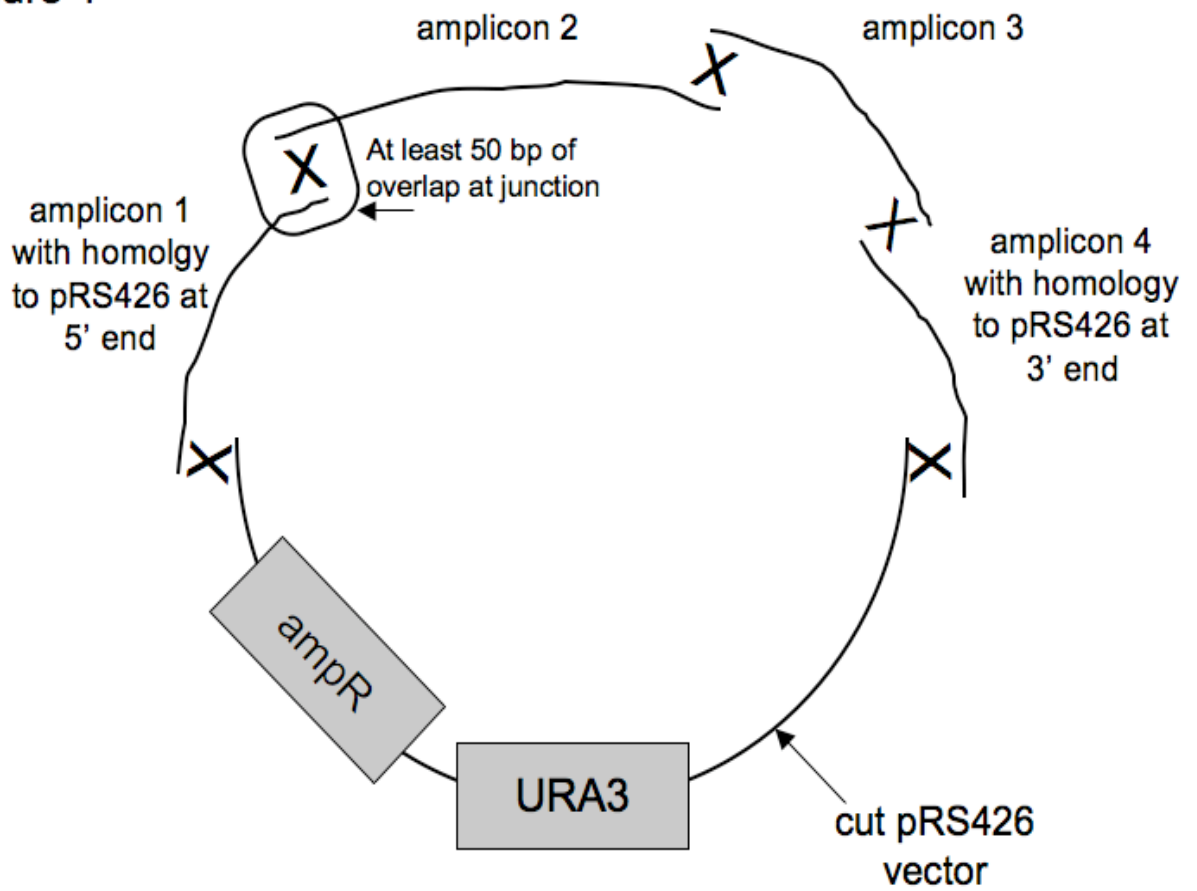
1% SDS

100 mM NaCl

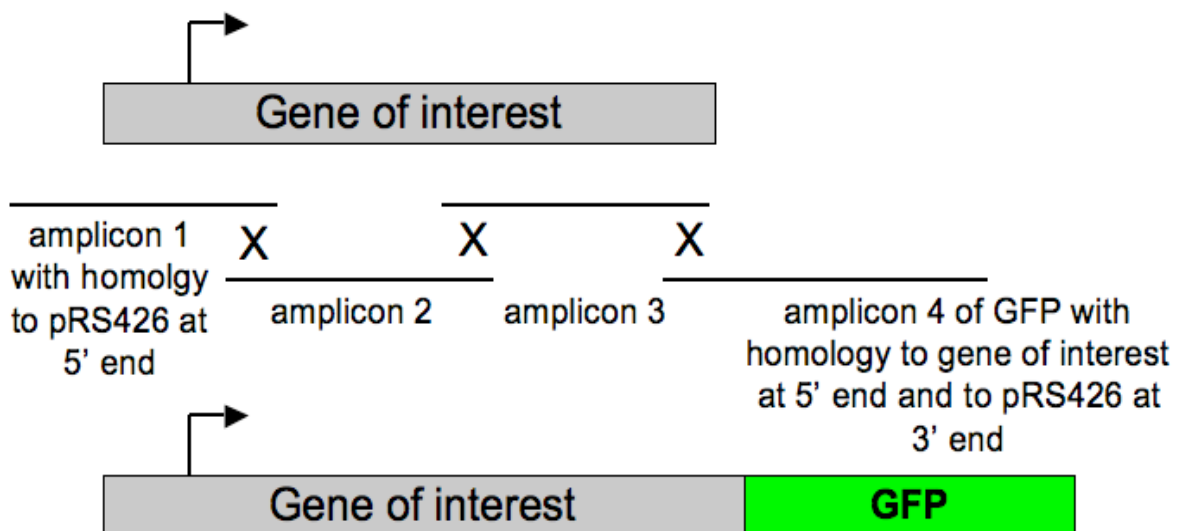
10 mM Tris pH 8.0

1 mM EDTA

Figure 1



For example:



The final product will have GFP inserted into the sequence.