Fusing GFP to the Carboxy Terminus of Your Favorite Protein

●Design the oligonucleotide primers for polymerase chain reaction (PCR)

The same primers used for the pFA6a plasmid can be used for pDH3 and pDH5. See Wach et al., Yeast Vol. 13: 1065-1075 (1997) for a discussion of primer selection.

We typically use ~60 mer PAGE-purified oligos with ~40 bp homology to the gene of interest.

Forward primer (5' to 3'):
(~40 bp upstream of stop codon)-GGTCGACGGATCCCCGGG

Reverse primer (5' to3'):
(~40 bp downstream of stop codon)-ATCGATGAATTCGAGCTCG

● Amplify the integration cassette form the plasmid

A polymerase with relatively high fidelity and high activity is necessary. The Roche Expand Long Template PCR system has worked well for us. The following protocol assumes use of the Expand system.

We find it helps to linearize the plasmid with *Not*I before using it as a template for PCR. Only a 15 minute digestion is necessary.

For a 50 μl of PCR reaction, make two solutions:

Solution 1:

Reagent	**Volume (μl)**
75 ng/μl linearized plasmid	1
100 pmol/μl forward primer	0.5
100 pmol/μl reverse primer	0.5
2.5 mM dNTP (2.5 mM **each**)	10
dH_2O	13

Solution 2:

Reagent	**Volume (μl)**
Buffer #3 (provided)	5
dH_2O	19.25
Expand polymerase mix	0.75

Mix solutions well individually. Combine and mix well again.

Run using the following PCR conditions:

94˚C for 2 minutes
92˚C for 10 seconds
50˚C for 30 seconds
68˚C for 4 minutes

Repeat previous three steps 9 times. Then,

92˚C for 10 seconds
50˚C for 30 seconds
68˚C for 4 minutes + 20 seconds per cycle

Repeat previous three steps 18 times. Then,

68˚C for 7 minutes
4˚C indefinitely

Run 5 μl on gel to confirm PCR.

● **Transform yeast with cassette**

1. Grow a 10 ml culture of diploid cells to ~90 Klett units (mid-log phase).
2. Pellet cells at 4˚C at 5,000 x g for 5 minutes. Decant supernatant.
3. Wash cells with 5 ml of dH_2O.
4. Repeat step 2.
5. Resuspend cells in 100 μl of 100mM lithium acetate (LiOAc).
6. Transfer to two Eppendorf tubes.
7. During cell preparation, boil sheared salmon sperm carrier DNA for 5 minutes and then place on ice for at least 2 minutes.
8. Pellet cells in microfuge for 15 seconds. Decant supernatant.
9. Add IN THIS ORDER to one tube:
    a. 240 μl for 50% PEG (mol. wt. 3350)
    b. 36 μl of 1.0 M LiOAc
    c. 25 μl of sheared salmon sperm DNA (10 mg/ml stock solution)
    d. 45 μl of cassette (from PCR above)
10. Mix. Place in 30˚Crotator and leave for 45 minutes.
11. Heat shock in 42˚C water bath for 25 minutes.
12. Pellet at 6,000 x g for 1 minute.
13. Resuspend cell in 100 μl of dH2O.
14. Plate mix on 2 YPD plates and incubate at 30˚C overnight.
15. Replica plate to the appropriate selective medium (YPD+G418, YPD+HygB or SD-his) the following day.
16. After 2 days at 30˚C, pick large colonies and streak for single colonies on selective medium.

Note: If transformation efficiency is low, try doubling the amount of carrier DNA or replacing your stock. In our experience, the carrier DNA is crucial for efficient transformation.

Finally one should confirm the correct integration by PCR.