Design

Gene knockout, developed in the 1980s, is a new molecular biotechnology based on gene homologous recombination. It is more and more popular to use it to study certain gene function or to construct unique animal model. In our experiment, we applied the fusion PCR to knock out the Gpa1 and Far1 genes. Fusion PCR is a rapid method which would generate linear fragments that can be used for transformation without having to use conventional cloning procedures and when the linear fragments integrate by homologous recombination into the genome they cannot easily excise. In detail, we need to design six primers to amplify three linear fragments of which two fragments are from S.cerevisiae genome flanking the third fragment as the selection cassette. Shown as the figure1, when we knock out the Far1 gene, flanking DNA fragments are amplified with primer p1 and p2 and with p5 and p6. Primer p3 and p4 amplify the selectable marker, zeocin, which must not be S.cerevisiae gene. Next, the two flanking DNA fragments and selection cassette are mixed and fused together by PCR using primer p1 and p6 when the Far1 has been replaced by zeocin. Finally, the fused fragment will integrate into the S.cerevisiae genome by homologous recombination during the transformation, as description of figure 2. Successful knockout yeast can be screened by plates containing zeocin. The same design concept applies to deletion of Gpa1 gene except the selectable marker, which changes into hygromycinB.

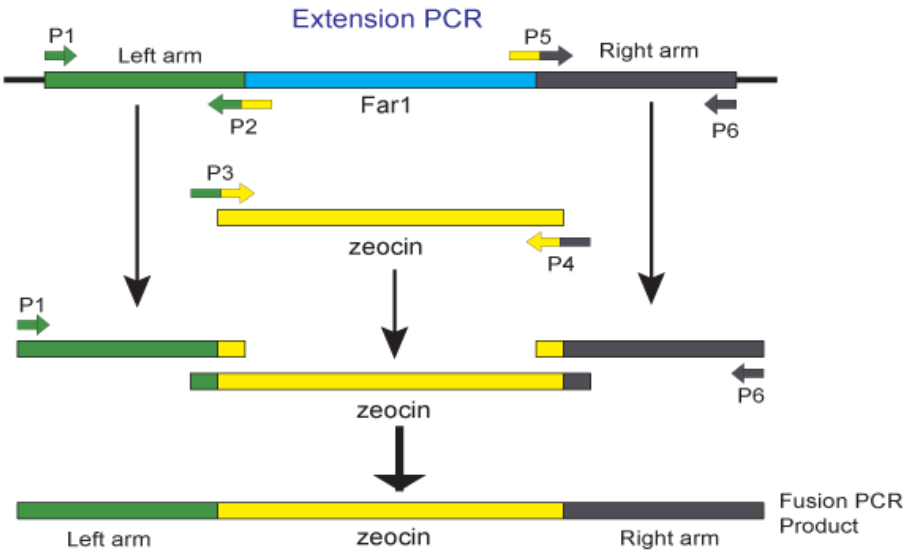

Figure 1: Sep up twice PCR to get the final fragment for tranformation.

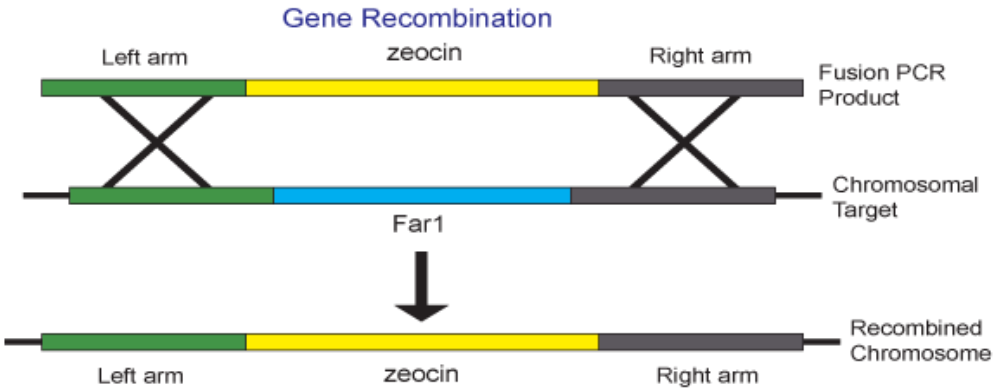

Figure 2: Fusion PCR product is transfromed into the S.cerevisiae resulting that the Far1 gene in yeast genome is replaced by zeocin.

Method

1. Design primers.
   Design six primers (p1-p6) as shown in the figure 1.We normally use homologous flanking regions about 1000bp in length in order to enhance knockout efficiency, so p1 and p2 would normally be about 1000bp apart, as would p5 and p6.The green portion of the p2 is identical to the antisense strand of the 3' end of left homologous arm and its red portion is same to 5' end of the strand of zeocin. In simple terms, the same color presents the same or complementary sequence. Therefore, the p2 and p3 would have 20 bp sequence complemented, as would p4 and p5. As for the rule of design, if possible, base composition of primer should be 50%-60% G+C and should not be long runs of A+T or G+C. The 3' terminal base should be G or C, but runs of three or more Gs and Cs at 3' terminus should be avoided. The primers should not form dimers or hairpin structures. We use the pimer primier 5 computer program to design and analyze the six primers which is used to knock out Far1, as outlined below.

Primers	Sequence
P1	AACGCACGCATGGAAAACAC
P2	GGTGTGTGGGTTGGGACGGATGGACAACTT
P3	TCCGTCCCAACCCACACACCATAGCTTCAAAATG
P4	GTCTACGTGTAGCTTGCAAATTAAAGCCTTCG
P5	TTTGCAAGCTACACGTAGACTCCACAACCTTTTC
P6	TTCAACATGCAGCCATTTCA

2. Set up two separate PCRs, one for each flanking genomic fragment before that we need to find the optimum conditions of the primers by PCR. The PCR use the taq enzyme in the PCR mix as DNA polymerase. Reactions are set up in tubes appropriate for PCR machine that will be used. We normally use 200ul thin-wall PCR tubes. Components are mixed on ice before the tubes are transferred to the PCR machine. When the optimum conditions are determined, we set up 50ul system of PCR using KOD FX as the DNA polymerase because the KOD FX

enzyme has higher fidelity of DNA replication. Primer p1 and p2 are used to amplify one fragment and p5 and p6 the other. We normally use QIAquick Gel Extration Kit to purify DNA, which can obtain more pure product than QIAquick PCR purification kit if there are nonspecific bandings.

3. Amplify the selection cassette from plasmid which contains Far1 gene, using the same reagents and conditions as are used for amplification of the genomic fragments except that the primers change into p3 and p4. After purification, the two fragments and the cassette are stored in -20℃.
4. Set up fusion PCR to produce the linear molecule for transformation. The template consists of two amplified flanking sequences and the cassette and the three parts must have equal mole number. Amplification using primer p1 and p6 fuses the three fragments into a single molecule. Purify the final product with the QIAquick gel extraction kit.
5. Use the T4 ligase to connect the final fragment with the PMD 20-T vector for 12h. It is important to note that the final fragment has to add "A" at the 3' end and 5' end of the sequence so that it can connect to T vector. Add "A" procedure normally needs rTaq enzyme and dNTPs which are contained in the PCR mix, so we mix the same amounts of the final fragment and 2×PCR mix to achieve it.
6. After 12h, transform the linked product to E.coli, top10, which is cultured on LB solid medium containing ampicillin at 37℃ for 12h. Then inoculate the single colony into 7ml LB liquid medium plus ampicillin in a 50ml centrifuge tube with shaking at 250 r.p.m for 12h at 37℃。
7. Extract the plasmid from the top10 by Plasmid Miniprep kit and check it by agarose gel electrophoresis after digest the plasmid using enzyme NdeI and BamHI.
8. Through analyze the agarose gel figure which is not show here, we can conclude that the final fragment linked to the PMD 20-T vector successfully. Next, set up a PCR using primer p1 and p6 to obtain the final fragment and these steps are used to make the final fragment more pure.
9. Transform the final fragment to S.cerevisiae, BY4741, by electroporation using MicorPulser system. It is reported that optimum conditions of electrotransformation of yeast is voltage of 1.5kV and time constant of ~5msec. The S.cerevisiae is spread on YPD solid medium containing zeocin and yeast need to grow on the medium for 3d to be seen. As for Gpa1 gene knockout, the selectable marker, hygromycinB, would degrade come across light, so every operation has to be protected from light.
10. Inoculate the single colony to another YPD solid medium plus zeocin for 3d again to remove the false positive colony.
11. Inoculate the single colony into 50ml YPD liquid medium plus zeocin in a 250ml Erlenmeyer flask with shaking at 250 r.p.m on a gyratory shaker for 12-16h at 30℃ with avoiding light.
12. Extract the yeast genome by Yeast Genome Extraction kit. Then set up a PCR using primers p1 and p6 followed by agarose gel electrophoresis to validate the Far1 in the genome is replaced by zeocin.

Additionally, the protocol of the knockout of the Gpa1 gene is similar to the Far1.The six primers sequence of knockout Gpa1 gene are shown as below.

Primers	Sequence
P1	TGAGCCTTTAACATTATCCCTT
P2	CATGCAAGCTGATAGTCACCGATCTAATCATCCAG
P3	TTAGATCGGTGACTATCAGCTTGCATGCCGGTCGATCTAGTAAC
P4	AATGATGTCATCGATCATACATGAGAATTAAGGGAGTCACGTTATGACCCCGC
P5	TTCTCATGTATGATCGATGACATCATTGGCTCTT
P6	GAACTAGATTTGGTCACAGACATGT