Short Protocols for Yeast Workshop

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Yeast Laboratory Course

Sanom Nonklang
Kamonchai Cha-aim
Rinji Akada

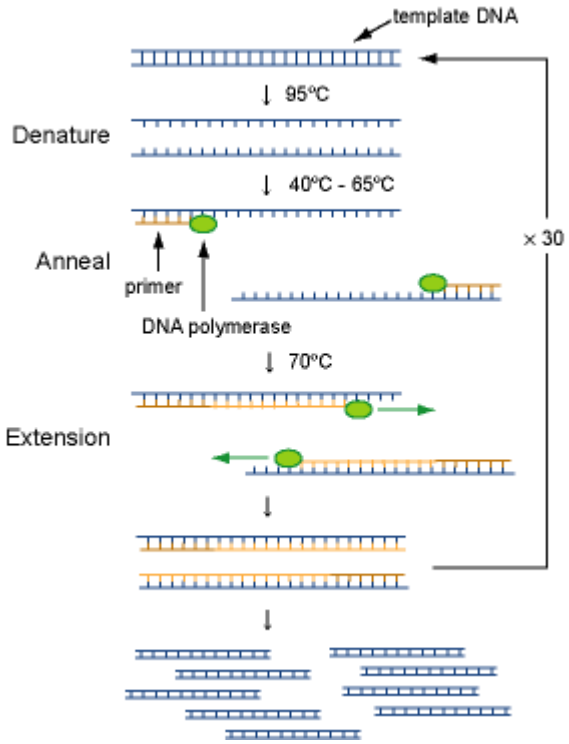
Department of Applied Molecular Bioscience
Yamaguchi University Graduate School of Medicine
Yamaguchi University
Japan

Experiment I: Fusion PCR

Kamonchai Cha-aim

Introduction

Polymerase chain reaction (PCR): The PCR is used to amplify a precise DNA fragment from template DNA. The PCR consists of three defined sets of cycle: denaturation, annealing and extension. The cycle is repeated 30-40 times, termed cycles. At first, the double-stranded template DNA is denatured by heating to above 90℃-95℃ (denaturation). The temperature is then cooled to between 40℃- 60℃ (annealing). The annealing step allows the hybridization of the two oligonucleotide primers, to bind to their complementary sites that in the target DNA. The DNA synthesis step is termed extension and is carried out by a thermostable DNA polymerase.



Because both strands are copied during PCR, there is an exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on.

Hot-start PCR allows the inhibition of polymerase activity during PCR reaction preparation. By limiting polymerase activity prior to PCR cycling, Hot-start PCR reduces non-specific amplification and increases PCR product target yield.

Fusion PCR：Gene fusion by PCR is a technique for recombinant DNA construction. For example, a gene of interest can be fused with marker gene or promoter. Recombinant DNA construction can be used directly for transformation into *S. cerevisiae* by homologous recombination. Fusion PCR, we recommend the poly C/G sequences. For example, 15C, 3C3G3C3G3C and 5C5G5C are fusion regions to connect two and three DNA fragments. These sequences were flanked by fusion primers to produce the overlap sequences in the first PCR products.

PCR condition (first round PCR)

A mixture of PCR reaction		PCR cycle		
Template DNA*	1 μl	Hot start	94℃	1 min
10 mM forward primer	0.2 μl	Denaturation	94℃	20 sec
10 mM reverse primer	0.2 μl	Annealing	60℃	30sec
2.5 mM $MgSO_4$	0.4 μl	Extension	68℃	1kb/1min
10X Kod Plus buffer	1 μl			
25 mM dNTPs	1 μl	(Hot start for one cycle; next follows 30 cycles of denaturation, annealing and extension)		
DW for PCR	6 μl			
Kod Plus polymerase	0.2 μl			
Total volume	10 μl			

*DNA concentration will be adjusted.

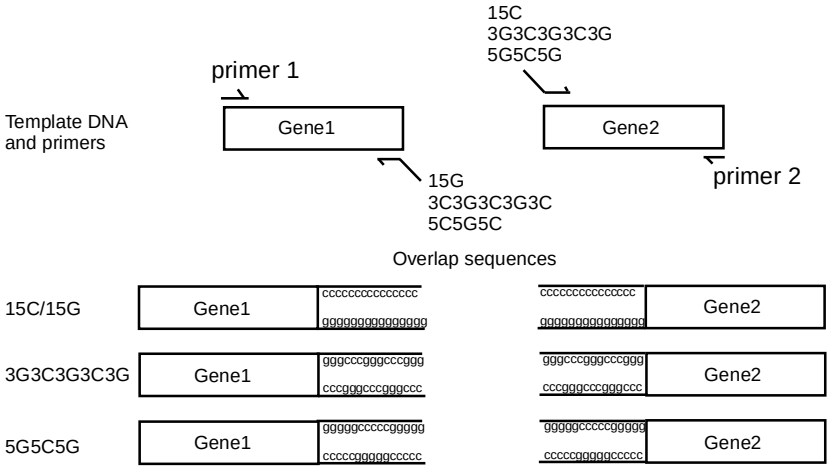


Figure1 Illustration of two gene fragments containing poly C/G overlap sequences for fusion PCR.

Steps of recombinant DNA construction by PCR

PCR product loaded into well of 0.7% agarose gel

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Electrophoresis

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Cutting DNA out from gel and DNA purification to eliminate primers

↓

DNA solution for fusion

PCR condition (second round PCR)

A mixture of PCR reaction

DNA fragment 1	0.5 μl
DNA fragment 2	0.5 μl
10 mM primer 1	0.2 μl
10 mM primer 2	0.2 μl
2.5 mM $MgSO_4$	0.4 μl
10X Kod Plus buffer	1 μl
25 mM dNTPs	1 μl
DW for PCR	6 μl
Kod Plus polymerase	0.2 μl
Total volume	10 μl

PCR cycle

Hot start	94℃	1 min
Denaturation	94℃	20 sec
Annealing	68℃	30sec
Extension	68℃	1kb/1min

(Hot start for one cycle; next follows 30 cycles of denaturation, annealing and extension)

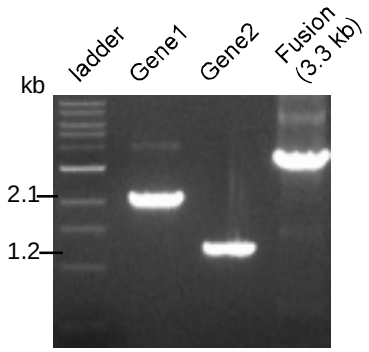


Figure2 Illustration of two DNA fragments fusion.

Experiment I: Construction of α-amylase gene (*TAA*) fused with *URA3* by using fusion PCR

The TAA gene encodes an α-amylase that belongs to *Aspergillus oryzea*. The α-amylase can degrade starch and show halo on YPD containing 1% starch which was reacted with iodine vapor. To construct recombinant DNA, TAA, marker gene and promoter are fused by fusion PCR and transformed into yeast. The steps of experiment are in this list below.

The steps of *TDH3p-TAA*-15G-*URA3* construction

Step I : Amplifications of *TAA* gene and *URA3* marker gene

- Amplification of TAA gene cassette by using chromosomal DNA of *S. cerevisiae* RAK3623 as template and primer pairs: URA3-200 and 15G-PGKterC
- Amplification of marker gene by using chromosomal DNA of *S. cerevisiae* BY4704 as the template and primer pair:15C-URA3-223 and URA3-200c

PCR condition (first round PCR)

A mixture of PCR reaction	
DW for PCR	6.6 μl
2.5 mM $MgSO_4$	0.4 μl
10X Kod Plus buffer	1 μl
25 mM dNTPs	1 μl
DNA template	0.4 μl
10 mM primer 1	0.2 μl
10 mM primer 2	0.2 μl
Kod Plus polymerase	0.2 μl
Total volume	10 μl

PCR cycle		
Hot start	94℃	1 min
Denaturation	94℃	20 sec
Annealing	50℃	30sec
Extension	68℃	3 min

(Hot start for one cycle; next follows 30 cycles of denaturation, annealing and extension)

- After PCR was completed, add 1 ul of gel loading dye to 10 μl of PCR product and then load 5 μl in 0.7% agarose gel, carry out electrophoresis at 100V, 30 min and excise DNA band from gel
- Put cut gel to DNA purification filter tip and then centrifuge 3 min at 12,000 rpm to obtain DNA solution.
- Mix DNA solutions with ratio 1:1 and then used as template for fusion PCR.

Step II: Fusion PCR_

A mixture of PCR reaction

DW for PCR	6 μl
2.5 mM $MgSO_4$	0.4 μl
10X Kod Plus buffer	1 μl
25 mM dNTPs	1 μl
DNA template	1.0 μl (mixure of fragment I and II)
10 mM primer 1	0.2 μl (URA3-200)
10 mM primer 2	0.2 μl (URA3-200c)
Kod Plus polymerase	0.2 μl
Total volume	10 μl

PCR cycle

Hot start	94℃	1 min
Denaturation	94℃	20 sec
Annealing	68℃	30sec
Extension	68℃	4.30 min

30 cycles

- Confirmation DNA fusion on 0.7% agarose gel

Result

Experiment II: Heterologous protein expression in *Kluyveromyces marxianus*

Heterologous protein used in this experiment is flocculin protein of *Saccharomyces cerevisiae*. Flocculin protein is involved in flocculation phenotype in yeast. Flocculin proteins are encoded by *FLO* genes. In *S. cerevisiae*, the five *FLO* gene families: *FLO1*, *FLO5*, *FLO9*, and *FLO11* have been described thus far. The *FLO1*, *5*, *9*, and *10* genes confer cell-cell adhesion (flocculation) ability, whereas *FLO11* is responsible for substrate adhesion. In this experiment we are going to express *S. cerevisiae FLO9* gene under regulation of *TDH3* promoter. Then, we can know whether expression of Sc*FLO9* can confer the flocculation phenotype in *K. marxianus*.

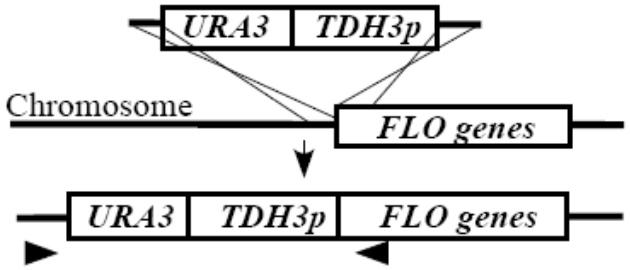


Figure 3. Construction of *URA3-TDH3p-FLO*

Transformation method (Stationary phase cell transformation)

Yeast culture

The yeast *K. marxianus* RAK3605 (*ura3-*) is used as a host. This strain is cultured in 25 ml YPD with shaking and incubated at 28 °C for 18 h.

Materials

1) 60% PEG 3350: Polyethylene glycol3350 (sterilized by filtration)
2) 4 M LiAc: Lithium acetate (autoclave)
3) 1 M DTT: Dithiothreitol in 0.01 M NaOAc pH 5.2 (filtration, stored at –20ºC)
4) Sterilize distilled water
5) Selective medium: Uracil dropout plates (-U),
6) PCR product 1-5 μl

Procedure

1. Prepare One-Step Buffer

60% PEG	667	333
4M LiAc	50	25
1M DTT	100	50
Water	183	92
Total (µl)	1000	500

2. Transfer 10 ml of cultured yeast cells into 15 ml centrifuge tube, centrifuge at 5,000 rpm for 3 min, discard supernatant
3. Add 300 µl of one-step buffer to cell pellet, re-suspend and transfer cell suspension to 1.5 ml microcentrifuge tube, centrifuge at 12000 rpm for 5 sec and discard all buffer
4. Add 200µl of one-step buffer to cell pellet, mix cells by using pipette and vortex
5. Transfer 100 ul of cell suspension to new 1.5 ml microcentrifuge tube
6. Add PCR product 1-5 µl and mix
7. Incubate at 47 °C for 15 min
8. Add liquid –U medium to transformation mixture and mix
9. Spread 200 µl transformation mixture on –U plate
10. Incubate at 28 °C for 2-3 days

Flocculation test

Grow yeast cells in test tubes containing 5 ml YPD with shaking for overnight. The culture tube is vortexed vigorously for 1 min and allowed to settle. Cell sedimentation is then observed.

Result

Experiment III: gene disruption in *K. marxianus*

Gene targeting transformation is a useful tool for deletion of a particular gene (gene knock out) or for introducing gene of interest into a desire locus. Generally, the integration of DNA fragment requires the action of double strand break (DSB) repair mechanisms. In eukaryotes, two major pathways have been identified, homologous recombination (HR) and non-homologous end joining (NHEJ). In *K. marxianus*, the NHEJ pathway was likely to function for DSB repair mechanism and linear DNA random integration. Therefore, we disrupted *KU70* gene which known to play an importance role in NHEJ pathway to improve gene targeting efficiency in *K. marxianus*.

In this experiment we are going to compare gene targeting efficiency in KU70 and ku70- strains and study the effect of the length of homologous sequence on gene targeting efficiency.

Material and method

Strains, marker and target genes in this study

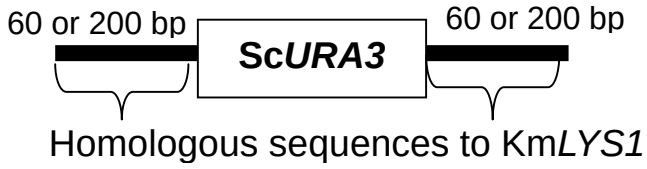
Host: *K. marxianus* RAK4174 (*ura3*$^{-}$ *leu2*$^{-}$ *KU70*$^{+}$*)*

K. marxianus RAK4736 (*ura3*$^{-}$ *LEU2*$^{+}$ *ku70*$^{-}$*)*

Target gene:Km*LYS1*

Marker gene :Sc*URA3*

Construction of Km*lys1*::Sc*URA3*



Transformation material

1) Test tube containing 2 ml of YPD
2) Petri dish containing 9 ml of YPD
3) 10 mg/ml Salmon DNA in TE buffer (Filtration, stored at -20°C) :
4) 60% PEG 3350 (filtration)
5) 4 M Lithium acetate (autoclave)
6) Sterilize distilled water
8) PCR product of desired gene(s) 5 μl : Kmlys1::URA3-60 and Kmlys1::URA3-200

fragments

9) Selective medium: Uracil dropout plates (-U)

10) Minimal medium (MM) supplemented with Leucine or supplemented with Leucine and Lysine

Transformation method

1. Inoculate a tooth pick full of yeast cells to 2 ml of YPD and then incubate at 28°C, 150 rpm for 18-24 hrs.
2. Transfer 1 ml of overnight culture into a pertridish containing 9 ml of YPD and then cultured at 28°C, 150 rpm for 5 hrs.
3. Prepare 1 ml transformation buffer by mixing 784 µl of 60%PEG3350, 59 µl of 4 M lithium acetate and 157 µl of DW.
4. Precipitate cells by centrifugation at 5,000 rpm for 3 min and then washed once with 300 sterilize DW. The pellet is re-suspended with 160 µl of TF buffer
5. Boil carrier DNA for 5 min and then quick cool on ice
6. Transfer 85 µl of cell suspension into microcentrifuge tube
7. Add10 µl of carrier DNA
8. Add 5 µl of desired PCR product.
9. Mix transformation mixture by vortexing for 30 sec
10. Heat shock at 42°C for 30 min.
11. Spread 200 µl of transformation mixture on a –U plate and then incubate at 28°C for 2-3 days.
12. Count the number of colonies appeared on –U plate and record
13. Replica plated on –U, MM+Leu and MM+Leu+Lys

Calculation of transformation efficientcy

$$\text{Transformation efficiency (CFU/µg DNA)} = \frac{\text{Number of transformants}}{\text{Amount of DNA (µg)}}$$

Calculation of gene targeting efficiency

$$\text{Gene targeting efficiency (\%)} = \frac{\text{Number of cells fail to grown on MM+Leu}}{\text{Total number of transformants}} \times 100$$

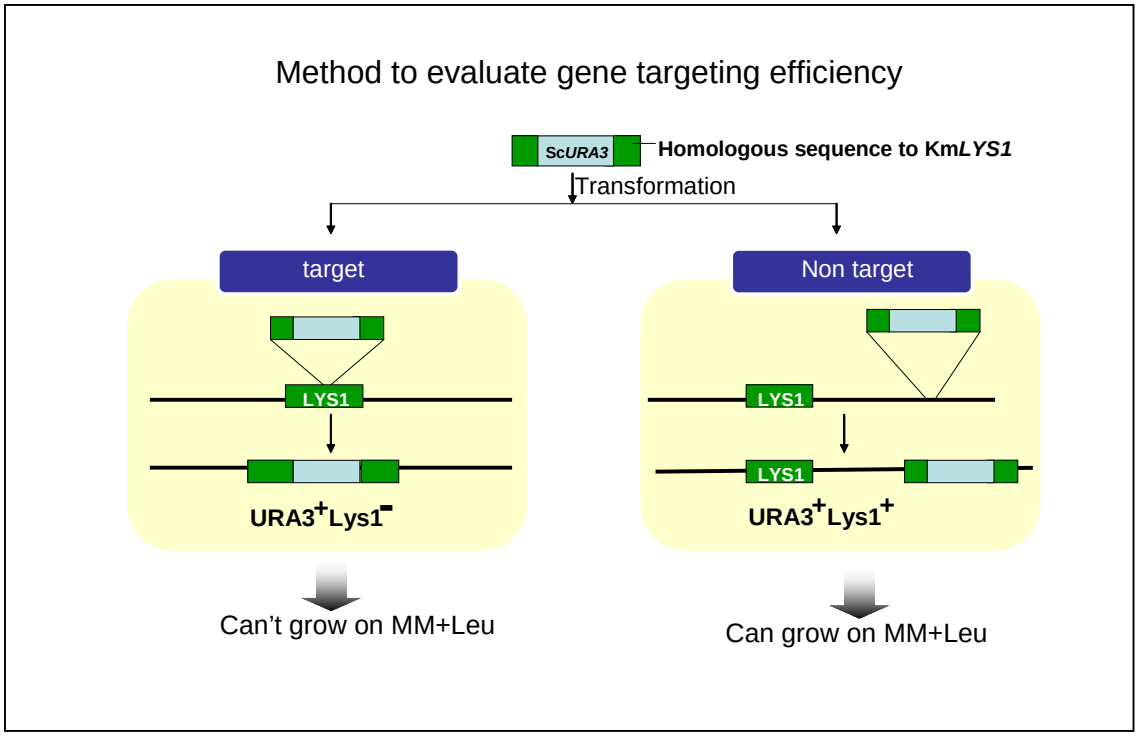


Figure 4. Method to evaluate gene targeting efficiency

Results

Transformation efficiency

Host	Transformed fragment	Amount of DNA (μg)	No. of transformants (CFU)	Transformation efficiency (CFU/ug DNA)
RAK4174	Kmlys1::URA3-60			
	Kmlys1::URA3-200			
RAK4736	Kmlys1::URA3-60			
	Kmlys1::URA3-200			

Gene targeting efficiency

Host	Transformed fragment	No. of transformants fail grow on MM+Leu	Total transformants (CFU)	Gene targeting efficiency (CFU/ug DNA)
RAK4174	Kmlys1::URA3-60			
	Kmlys1::URA3-200			
RAK4736	Kmlys1::URA3-60			
	Kmlys1::URA3-200			

Appendix

1. Stock preservation

Yeast strains can be stored for short periods of time at 4℃ on YPD medium in Petri plates or in closed vials (slants). Although most strains remain viable at 4℃ for at least 1 year, unusually sensitive mutants die after several months. Yeast strains can be stored indefinitely in 15% (v/v) sterile glycerol at －60℃ or lower temperature.

How to preserve yeast?

The strains are first grown on the surface of YPD plates; the yeast is then picked up with sterile applicator sticks and suspended in the glycerol solution. The caps are tightened and the vials shaken before freezing.

2. Media

For experimental purposes, yeast are usually grown at 30℃ on the complete medium, YPD, or on synthetic media, minimal (SD). Synthetic media are prepared with Bacto-yeast nitrogen base without amino acids (see synthetic dextrose minimal media). Growth on nonfermentable carbon sources can be tested on YPG medium. Media for Petri plates are prepared in 2-liter flasks, with each flask containing no more than 1 liter of medium, which is sufficient for approximately 40 standard plates. All component are autoclaved together for 20 min at 121℃ and 15 pounds pressure. The plates should be allowed to dry at room temperature for 2-3 days after pouring. The plates can be stored in sealed plastic bags for over 3 months at room temperature.

YPD (for routine growth)	
Yeast extract (1%)	10 g
Peptone (2%)	20 g
Glucose (2%)	20 g
Agar (2%)	20 g
Distilled water	1000 ml

YPG	
Yeast extract (1%)	10 g
Peptone (2%)	20 g
Glycerol (3% v/v)	30 ml
Agar (2%)	20 g
Distilled water	970 ml

YPD 1% starch	
Yeast extract (1%)	10 g
Peptone (2%)	20 g
Glucose (2%)	20 g
Starch soluble (1%)	10 g
Agar (2%)	20 g
Distilled water	1000 ml

Minimal medium (MM)

Yeast nitrogen base*	1.7 g
$(NH_4)_2SO_4$	5.0 g
Glucose (2%)	20 g
Agar (2%)	20 g
Distilled water	1000 ml

* without amino acid and ammonium sulphate

Minimal medium supplemented with uracil (MMU)

MMU is MM supplemented with uracil 20 mg/liter

Drop out media

In order to test the growth requirements of strains, it is useful to have media in which each of commonly encountered auxotrophies is supplemented except the one of interest (droup-out media). Dry supplements are stored premixed.

Drop out mix

Amino acid	**-U**	**-W**	**-H**	**-L**	**-K**
Adenine hemisulfate	0.5 g	0.5 g	0.5 g	0.5 g	0.5 g
Uracil	-	2.0 g	2.0 g	2.0 g	2.0 g
L-Tryptophan	2.0 g	-	2.0 g	2.0 g	2.0 g
L-Histidine HCl	2.0 g	2.0 g	-	2.0 g	2.0 g
L-Methionine	2.0 g	2.0 g	2.0 g	2.0 g	2.0 g
L-Leucine	4.0 g	4.0 g	4.0 g	-	4.0 g
L-Lysine HCl	2.0 g	2.0 g	2.0 g	2.0 g	-
Total	12.5 g	12.5 g	12.5 g	10.5 g	12.5 g
Per liter	0.6 g	0.6 g	0.6 g	0.5 g	0.6 g

Uracil droup out medium (-U medium)

Drop out mix (-U)	0.6 g
Yeast Nitrogen Base*	1.7 g
$(NH_4)_2SO_4$	5.0 g
Glucose	20 g
DW	1 L
Agar	20 g

* Without amino acids and without ammonium sulfate