# Materials

**Yeast strain**

A highly transformable *S. cerevisiae* strain VL6-48 (MAT alpha, his3-D200, trp1-D1, ura3-52, lys2, ade2-101, met14, psi+cir0), in which HIS3 and TRP1 genes has been deleted, is used as a host for gene cluster direct cloning experiments. This strain is available from the American Type Culture Collection (ATCC no. MYA-3666).

In adenine insufficient medium, this strain forms pinkish colonies (addition of adenine makes their color creamish). For spheroplast preparation, the yeast cells are grown in liquid YPD medium (Yeast extract peptone dextrose medium; 2% D-glucose, 1% yeast extract, and 2% peptone) supplemented with 100mg/l adenine (addition of adenine is not necessary for their growth but is recommended for the direct cloning experiments).

*S. cerevisiae* strain VL6-48 cannot grow in Trp deficient media due to deletion of TRP1 gene. Therefore, the TRP1 gene is used as a selectable marker. For this strain, the HIS3 gene can also be used as a selectable marker. Use of the URA3 gene as a marker for the direct cloning experiments is not recommended, even though the strain is the URA3 gene deficient (the URA3 gene on its chromosome is inactivated but not deleted, and thus unwanted recombination occurs at relatively high frequency between chromosomal URA loci and the URA gene on the vector).

Grow them on YPD agar plate supplemented with 100mg/l adenine and store the plate in 4℃ until use (the *S. cerevisiae* VL6-48 colonies on YPD plate can be stored in 4℃ for a month).

**Yeast transformants selective agar (Trp deficient)**

*10x nitrogen bases*

1.7g of Yeast Nitrogen Base Without Amino Acids and Ammonium Sulfate (SIGMA Y1251)
1.9g of Yeast Synthetic Drop-out Medium Supplements without tryptophan (SIGMA Y1876)
5g of Ammonium Sulfate
100mg Adenine (Make adenine separately in 100x stock solution and filter sterilize. Add 1 equivalent of HCl to help it dissolve)

Disolve above components in 100ml warmed up Milli-Q water and filtrate with 0.22um filter to make x10 stock sol (store at 4℃).

Autoclave mixture of 1.1M sorbitol, 2.2% glucose, and 2.2% agar, and then mix it with 10x N-bases to make selective agar.

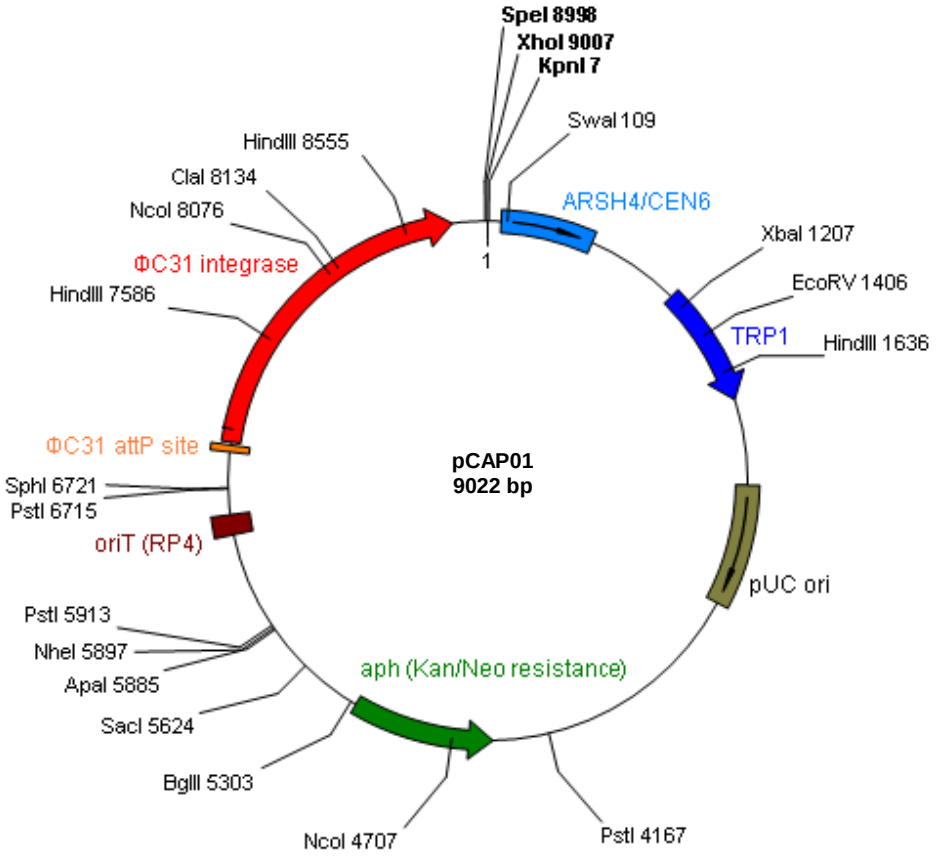
**Capture vector pCAP01** (A yeast centromeric-*E.coli* shuttle and Actinobacterial chromosome integrative)

This vector has ARSH4/CEN6-TRP1 (yeast elements from pRS314), pUCori from SuperCos1 (for maintenance in *E.coli*), and $\phi$C31 *int-attP-oriT- aph* (*Streptomyces* elements from a pSET152 derivative). For yeast, Trp deficient media is used for selection of transformants. Both in *E.coli* and Actinobacteria, 50ug/ml Kanamycin (or 30-50 ug/ml Neomycin) is used as a selective agent. If you need to use a expression host that has *aac(3)IV* (apramycin resistance) gene already or a Kanamycin resistant strain, use 30-50 ug/ml Neomycin for the selection.

This vector is maintained at single copy in yeast cells. This is very important feature to avoid unwanted multiple recombination in yeast. So far, it has been confirmed that huge DNA insert up to 75kb could be cloned and carried stably on this vector (both in yeast and *E.coli*).

To construct a pathway specific capture vector, specific capture arms for the gene cluster of interest should be inserted into pCAP01 (*SpeI-XhoI-KpnI* sites are available). Minimum size of the arms is 60bp. However, longer arm (~1kb) may be more efficient. Before spheroplast transformation, pathway specific vector should be linearized (gel purification is recommended to remove undigested circular form of the plasmid perfectly).

A new capture vector, pCAP02, equipped with $\phi$BT1 integration system ($Apr^r$) is also available.



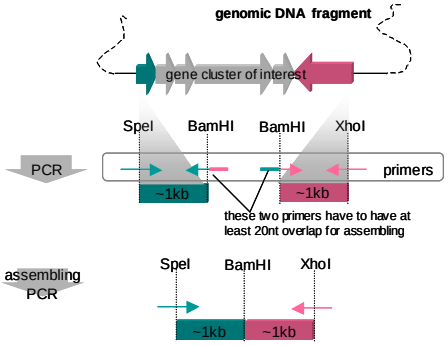

| **FEATURES** | Location/Qualifiers |
|---|---|
| **source** | **4478..8997** |
| | **/note="Cloning vector pLAE101, a pSET152 derivative"** |
| rep_origin | 6460..6571 |
| | /note="RP4 origin of single-stranded DNA transfer" |
| | /direction=RIGHT |
| misc_feature | 6919..6957 |
| | /note="phiC31 attP site" |
| gene | 6974..8815 |
| | /gene="int" |
| CDS | 6974..8815 |
| | /gene="int" |
| CDS | complement(4478..5272) |
| | /gene="aph" |
| **source** | **2127..4477** |
| | **/note="E.coli element from SuperCos1"** |
| rep_origin | 2278..2955 |
| | /label=pUC ori from SuperCos1 |
| **source** | **13..2126** |
| | **/note="pRS314"** |
| gene | 1124..1798 |
| | /gene="TRP1" |

```
CDS             1124..1798
                /label="ORF frame 3"
misc_feature    74..592
                /label="CEN6_ARS4"
```

# Procedure

**Construction of pathway specific capture vector** (in the case of 1kb capture arms)

PCR amplify 1kb regions from both ends of the target gene cluster. Each PCR primer must have appropriate restriction sites for the pathway specific vector construction (see below example).




The PCR amplified left side arm (in green) may have *SpeI* restriction site on its left and an unique restriction site (for example, *BamHI* site) on its right. In the same way, the right side arm (in pink) may have *XhoI* restriction site on its right and the same unique restriction site on its left. Two primers with the unique restriction site (linker region of the assembly) must have at least 20nt overlapping sequence for following PCR assembling. Mix two 1kb PCR fragments (purified) and assemble them into single piece by PCR with primers for both ends (use of PrimeSTAR HS polymerase with GC buffer is recommended. See http://www.clontech.com/takara/US/Products/PCR_Products/High_Fidelity_PCR/PrimeSTAR_HS_with_GC_Buffer?unitid=U100005928). Digest the assembled fragment and insert it into *SpeI-XhoI* site (*KpnI* site can also be used) on pCAP01 to generate your pathway specific capture vector. When you digest pCAP01 also add CIP (cow intestine phosphatase) to prevent recircularization when you ligate the 2 kb into it.Amplify the construct in *E.coli* (you will need the construct up to 2ug for every single yeast transformation). Before use, the vector must be linearized by restriction digestion with the designated enzyme (*BamHI* etc.).

**Preparation of genomic DNA**

In yeast spherolast transformation step, cells are transformed with linearized capture vector and gDNA fragments. gDNA must be fragmented to appropriate size because too large fragment is hardly taken up by the cells. Quality of gDNA fragments (especially in size distribution) also greatly affects recombination efficiency. Protocol described below gives you nicely purified gDNA fragments.

1. Resuspend mid- to late-log phase actinobacterial cells from 2-4 ml culture into 450ul of Sol.1 (10% sucrose, 50mM Tris-HCl (pH8.0), 10mM EDTA) in 2ml MCT.
2. Add 50ul of 30mg/ml lysozyme dissolved in Sol.1 and mix well. Incubate for 1h at 37℃ with shaking.
3. Add 10ul of Proteinase K (5mg/ml) and then add 150ul of 3.3% SDS. Invert the tube gently a few times and incubate the tube for 1h at 37℃ (without shaking). After incubation, cells must be lysed completely. If you still see flocs, expose the tube to 50℃ for additional 15-30 min.
4. Transfer the sticky lysate to 2ml *phase lock gel heavy* tube (http://www.5prime.com/products/nucleic-acid-purification/organic-nucleic-acid-extraction/phase-lock-gel-.aspx) by decantation.
5. Add 500ul PCI to the viscous cell lysate and shake the tube 5~10 times violently to make evenly mixed emulsion, then vortex the tube for 30 sec at maximum speed.
6. Spin the tube for 5min at maximum speed.
7. Decant upper phase into new 2ml MCT and add 700ul of IPA.
8. After inverting the tube a few times, you will see visible DNA floc. Invert the tube multiple times until jelly-ish thing is disappeared.
9. Remove supernatant carefully by pipette tip (no need to spin).
10. Add 1ml of 70% EtOH and mix. Then remove supernatant completely (keep DNA wet).

11. Add 400-500ul of TE containing RNase A (final 0.1mg/ml) to the DNA pellet (do not mix!). Keep the tube overnight in fridge or at 37℃ for 2h to allow dissolving gDNA completely.
12. Warm the DNA up at 50℃ and mix the DNA gently by pipetting with wide-bore tip.
13. Re-purify the DNA by EtOH precipitation.
14. Add 400-500ul of 0.2X TE onto the resultant DNA pellet and incubate at 37-50 ℃ for 2h to allow dissolving gDNA completely.
15. Check that the fragment size is >50-KB on agarose gel by comparison with λ-DNA markers (0.7% gel, 20V / overnight). Typical yield of gDNA from 2ml culture is 100-200ug.
16. Digest gDNA overnight with a restriction enzyme that doesn't cut your target gene cluster but cuts its flanking regions. In case of gDNA from high-GC organisms, enzymes recognizing AT-rich sequences such as EcoRI, HindIII, SpeI, NdeI, NheI, and XbaI may be used (if there is no enzyme generating nicely fragmented gDNA, try with partial digestion).
17. Clean up digested gDNA by EtOH precipitation.
18. Dissolve DNA fragments in 100-200 ul of 0.2x TE.
19. Measure the concentration of the DNA.
20. Keep it in –20℃ until use (gDNA fragments prepared by this way is now ready for transformation).

**Preparation of yeast spheroplast cells and transformation**

In prior to transformation, prepare below reagents.

**SPE**; 1 M sorbitol, 10 mM HEPES (pH 7.5), 10 mM EDTA (filtrate and store at room temperature).

**STC**; 1 M sorbitol, 10 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ (filtrate and store at room temperature).

**SOS**; 1 M sorbitol, 6.5 mM $CaCl_2$, 0.25% (w/v) yeast extract, 0.5% (w/v) peptone (filtrate and store at room temperature).

**20% PEG**; 20% (w/v) PEG 8000, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5 (filtrate and store up to 2 weeks at room temperature).

**Zymolyase 20T**; 10 mg/ml zymolyase-20T (MP Bio), 25% (w/v) glycerol, 50 mM Tris-HCl (pH 7.5). Dispense into 500-µl aliquots, and store up to 2 years at −20℃.

**1M sorbitol** (Autoclave and store at 4℃)

**MilliQ** (Autoclave and store at 4℃)

**Yeast transformants selective agar**; see above.

**Yeast transformants selective top agar**; same as selective agar but have 3% agar. After autoclave, dispense 7ml into 15ml tubes. Put the tubes into 60℃ water bath until use.

Current protocol below has been slightly modified from the original method descrided in Nture Protocols.

While they prepare yeast spheroplast cells from mid-log phase cells (OD600=3.0-5.0), early-log phase cells (OD600<1.0) are used in the modified protocol below since younger cells show higher competency.

To transform yeast cells with huge DNA molecule such as genomic DNA, cell wall must be removed perfectly by a lytic enzyme, Zymolyase, in prior to transformation. However, too much Zymolyase treated cells are hardly regenerated because spheroplast cells are extremely unstable. The original protocol doesn't have osmotic stabilization step before spheroplasting, and thus they could digest cell wall only partially with the enzyme (<80% conversion). By adding stabilization step before spheroplasting, Zymolyase treatment can be more perfectly done (nearly 100% conversion) without cell lysis. By these modifications, now we can transform yeast cells with huge DNA molecules at a high efficiency.

Basic transformation protocol

Day 1

1. Inoculate single colony of *S. cerevisiae* strain VL6-48 grown on YPD agar supplemented with adenine (100mg/L) into YPD plus adenine liquid media (~10ml) and grow them overnight at 30 ℃ with shaking. Colonies grown on YPD pates can be kept at 4℃ for up to 1 week.

Day 2

2. Transfer 2 mL of fully-grown pre-culture to fresh 100ml YPD plus adenine media (in 500ml-flask) and grow them with shaking until OD600 of 0.7-1.0 is reached (it takes 5 to 8h). This will give you enough amounts (for 20 transformations) of nicely grown early-log phase cells (growth phase of the cell greatly affects transformation efficiency).
3. Put the flasks on ice for 10min and then centrifuge the cells in a 50-ml centrifuge tubes for 3 min at 1800×g, 4℃.
4. Remove the supernatant, and resuspend the cell pellets in 50 ml sterile water (4℃) by vortexing. Wash the cells by centrifuging 3 min at 1800×g, 4℃.
5. Remove the supernatant, and resuspend the cells in 50 ml of 1 M sorbitol (4℃) by vortexing. Leave the cells on ice (or 4℃) overnight to osmotically stabilize the yeast cells prior to forming spheroplasts.

Day 3

6. Invert the tube a few times and then centrifuge the cells in a 50-ml centrifuge tube for 3 min at 1800×g, 4℃.
7. Remove the supernatant completely by standing the tube upside down on a paper towel, and resuspend the cells in 20 ml SPE (room temperature) by vortexing. Add 40μl of 2-ME, and invert to mix. Then add 80μl zymolyase-20Tsolution and invert to mix.
8. Incubate 40 min in 30℃ incubator (invert the tube every 10 min or use shaker with slow speed).
9. Check the level of spherolasting by measuring the optical density (at 600nm) of cell suspension. Dilute zymolyase treated cells 5 times with 1M sorbitol and 2% SDS (in water). Measure optical density at 600nm of both suspensions and compare their ODs. The spheroplasting are determined to be ready when their difference is ten- to twenty fold (≒90-95% of cells has to be converted to spheroplast). If the difference is not enough, keep incubating for additional 10min and measure the OD difference again. The level of spheroplasting greatly affects transformation efficiency (insufficiently digested cells do not take up large DNA molecule, but incubation time shouldn't be longer than 50min).
10. Add 1 M sorbitol (4℃) up to 50 ml. Gently invert to mix, and harvest the cells by centrifuging for 10 min at 600×g, 4℃.
11. Decant the supernatant, and stand the tube upside down on a paper towel to remove supernatant completely (cell pellet is not tightly packed! Do not loose cells!).
12. Gently resuspend the spheroplasts in 20 ml of 1 M sorbitol (4℃), by pipetting up and down with a 25-ml pipette. It takes about 2 min to resuspend the spheroplast cells completely (spheroplast cells are extremely unstable. Do not use vortex to resuspend). Add 1 M sorbitol (4℃) to a total volume of 50 ml, and invert multiple times to mix.
13. Harvest the cells by centrifuging for 10 min at 600×g, 4℃. Remove supernatant completely by standing the tube upside down on a paper towel.
14. Resuspend the spheroplasts such that each transformation will use 200 μL of STC solution (room temperature) by pipetting up and down with a 10-ml pipette (it takes about 2 min to resuspend the spheroplast cells completely).
15. Incubate the spheroplasts 10 min at room temperature (22℃).
16. Mix 200 μl spheroplasts with the ≤40 μl transforming DNA solution (0.2-1.0ug linearized vector and 1-2ug gDNA.) already contained in a 2ml-microcentrifuge tube by slowly adding them to the DNA, while stirring with the pipette tip at the same time (use of wide-bore tip is recommended). Make a few transformations in parallel with gDNA fragments prepared by different enzymes.
17. Incubate the spheroplasts/DNA mixture 10 min at room temperature.
18. Add 0.8ml 20% PEG solution, and mix by inverting the tube 10 times.
19. Incubate the tube 20 min at room temperature.
20. Microcentrifuge the cells 10 min at 700×g, 4℃.
21. Remove the supernatant with a 1-ml pipette.
22. Add 800 μl SOS solution, and resuspend the cells by pipetting up and down with a wide-bore 1-ml pipette tip.
23. Incubate the tube for 30-40 min at 30℃.
24. Add cell suspension into 7ml melted selective top agar prepared in 15ml tube (kept at 60℃), and invert a few times to mix. Immediately pour cells onto selective agar plate.

25. Incubate the plates for 3-5 days at 30℃.

Use circular capture vector (~0.1μg) as a positive control. It will give you uncountable colonies, if spheroplast cells are competent enough. Cells harboring an empty capture vector can form visible colonies in 2-3 days, but cells carrying a large insert grow more slower.

Alternative transformation protocol (time saver version)

Day 1

1. Inoculate single colony of *S. cerevisiae* strain VL6-48 freshly grown on YPD agar supplemented with adenine (100mg/L) into 100ml YPD plus adenine liquid media and grow at 30 ℃ with shaking until OD600 of 0.7-1.0 is reached (12-16h).

Day 2

2. Put the flasks on ice for 10min and then centrifuge the cells in a 50-ml centrifuge tubes for 3 min at 1800×g, 4℃.
3. Remove the supernatant, and resuspend the cell pellets in 50 ml sterile water (4℃) by vortexing. Wash the cells by centrifuging 3 min at 1800×g, 4℃.
4. Remove the supernatant, and resuspend the cells in 50 ml of 1 M sorbitol (4℃) by vortexing. Leave the cells on ice (or 4℃) for 4h to osmotically stabilize the yeast cells prior to forming spheroplasts.
5. Follow the protocol #6 to #25 described above.

Isolation of candidate colonies

During 3-5 days incubation, yeast transformants form visible colonies on the selective agar plate. Many colonies result from undesired recombination, and thus they don't have your gene cluster of interest. To identify desired clones, you need to screen many colonies by colony PCR.

1. Pick up 100-200 colonies with toothpicks and transfer them onto new selective agar plates.
2. Incubate the plate for 2 days at 30℃.
3. Make three replica plates by using filter paper.
4. Incubate the plates for 1-2 days at 30℃ (the replica plate "1" is used for colony PCR screening of each column. The plate "2" is for screening of each row).

Colony PCR screening

Multiplex PCR amplifying several different regions in the cloned cluster at the same time is recommended.

1. Scoop cells from each column or row with loops or 200ul pipette tips, and resuspend them into 100ul of 10 mM Tris-buffer pH8 (1/1000 dilution) and 0.2mg/ml zymolyase 20T (in 200ul PCR tube).
2. Incubate for 2h at 30℃.
3. Boil for 5min (thermal cycler can be use, 98℃/5min).
4. Spin down boiled cells and dilute supernatant 10 or 100 times. Take 1 μL for PCRUse 0.2ul as a template for 20ul scale PCR reactions. Clones showing all desired PCR signals are chosen for DNA extraction.

**DNA extraction from PCR positive yeast clones**

1. Pick up identified clone from plate "3", and inoculate it into 3–5ml yeast selective liquid medium (without Trp). (10*N-base stock with 2% glucose, 0.22um filtration)
2. Incubate 20-30h at 30℃ with shaking.
3. Resuspend cells from 1ml of fully-grown culture into 200ul of SPE containing 2-ME (1/500 dilution) and 0.5mg/ml zymolyase (use rest of 1ml culture to make glycerol stock).
4. Incubate them for 1-2h at 30℃. (at least 1h, you can see the decrease of the OD)
5. Lyse cells completely by adding 200ul P2 (Qiagen) and gentle inverting.
6. Add 200ul of P3 (Qiagen, do not use N3) and invert the tube gently.
7. Leave the tube on ice for 10 min.(or just room temperature incubation)
8. Spin the tube for 15min at maximum speed and recover supernatant.

9. Remove residual proteins by phenol-chloroform denaturation, and then precipitate and clean up DNA by using IPA and 70% EtOH.
10. Dissolve DNA in 20-50ul of 0.2x TE, and use the DNA sol. for *E.coli* transformation.

**Maintenance of the construct in *E.coli***

The capture vector has the *aph* (Neomycin / Kanamycin resistance) gene on its backbone. Use 50ug/ml Kanamycin for the selection in both *E.coli* and *Streptomyces* expression host.

As described earlier, pCAP01 has pUC-ori for maintenance in *E.coli*. pUC-ori is a high-copy number rep origin (100-500 / cell) derived from pBR322 low-copy rep origin (~20 / cell). Therefore, enough amount of pCAP01-derived construct can be easily prepared from conventional *E.coli* cloning hosts.

From the point of view of plasmid stability, low-copy replication would be better in general. However, SuperCos1 utilizing pUC-ori can carry relatively large DNA fragment (~ 60KB) very stably. Likewise, it has been confirmed that pCAP01 capture vector equipped with pUC-ori from SperCos1 can also carry huge DNA fragment (at least up to 75KB). If your construct has insert larger than 75KB, some stability issues may happen in maintenance in *E.coli*. For propagation of large construct, use of *recA*- and *endA*- strain such as *E.coli* Top10 or *E.coli* strain Stbl4 (invitrogen, Genotype: *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1 *gyr*A96 *gal*- *thi*-1 *sup*E44 λ- *rel*A1 Δ(*lac*-*pro*AB)/F' *pro*AB+ *lac*IqZΔM15 *Tn*10 (Tet$^R$)) is recommended to avoid unwanted rearrangement of the cloned cluster. In the *recA*$^+$ and/or *endA*$^+$ *E.coli* strains such as BW25113 (for λ-red recombination) and ET12567 (used for conjugal DNA transfer), this stability issue tends to arise. If you encounter this, in order to increase its stability, copy number of your construct can be easily lowered (to original pBR322 level) by cultivating *E.coli* host cells at 30℃, since pUC-ori shows high copy number replication only at higher temperature than 30℃ (for example, at 42℃, it shows extremely high copy replication). By this copy number control strategy, relatively large constructs can be maintained stably in *E.coli*.

**DNA extraction from *E.coli* cells**

If the size of cloned cluster is smaller than 40KB, Qiagen plasmid extraction kit should work. Use warmed up 0.2x TE for elution. For extraction of huge construct, use traditional mini-prep method. Then check restriction patterns to confirm direct cloning of the gene cluster.

**Mutagenesis and conjugal transfer of the cloned cluster**

For mutagenesis of the cloned cluster, λ-red recombination can be used. However, you may encounter above mentioned stability and/or rearrangement issue if your target cluster is large enough, and thus it may be difficult to get correctly mutated cluster. If it is the case, in vivo yeast recombination can be used to do mutagenesis experiment instead of λ-red-mediated mutagenesis in *E. coli* (see below).

For conjugal DNA transfer to an expression host, regular method using *E.coli* ET12567 or S17-1 can be used if the cluster size is not too large (>50KB). For large cluster, grow *E. coli* cells carrying the cluster at 30℃. If you still have some difficulties to grow them, try tri-parental conjugal DNA transfer from *E.coli* Top10 carrying your target gene cluster (Stbl4 strain can not be used for this purpose because the strain is resistant to Nalidixic acid) with the help of *E.coli* ET12567/pUB307. In this genetic system, efficiency of conjugal transfer is quite low but still enough to get large cluster transferred to a heterologous expression host.

**Yeast in vivo recombination-mediated mutagenesis**

In λ-red mediated mutagenesis, a PCR amplified selective marker gene flanked by >39nt homologous sequences to the target region is used for gene replacement. Since yeast cells naturally have strong homologous recombination activity, same PCR targeting strategy can be used for in vivo yeast homologous gene replacement. The URA3 or HIS3 gene can be used as a selective marker in the strain VL6-48 (TRP1 gene has been already used for the gene cluster direct cloning).

Mutant cluster can be generated by transformation of VL6-48 cells carrying captured cluster on pCAP01 vector with PCR amplified URA3 or HIS3 gene flanked by >39nt homologous sequences. See detailed protocol below.

Preparation of electrocompetent cells

1. Inoculate *S.cerevisiae* VL6-48 carrying target gene cluster (from glycerol stock) into 3-5ml yeast selective liquid medium (without sorbitol and Trp), and grow overnight at 30℃ with shaking.

2. Transfer 2ml to 50 ml fresh selective liquid medium and grow at 30℃ until OD600 is reached to 1.0-2.0.
3. Put the flasks on ice for 10min and then centrifuge the cells in a 50-ml centrifuge tubes for 3 min at 1800×g, 4℃.
4. Wash the cells with 1M sorbitol (4℃) twice.
5. Resuspend cells in remaining drop (～200ul) and dispense 40ul cells into 1.5-ml-MCT.
6. Keep the tubes on ice until use (Cells can be kept at –80℃ for up to 6 month. Do not use liquid nitrogen to freeze cells).

Preparation of selectable marker (in case of URA3 gene from pGAL-MF vector)

Prepare PCR amplified URA3 gene with its promoter by following PCR condition.

Forward primer (5' to 3'); 39nt homology arm – cagattgtactgagagtgca (59nt)
Reverse primer (5' to 3'); 39nt homology arm – tgtgagtttagtatacatgca (60nt)
Template; pGAL-MF plasmid (>0.1ng for 20ul PCR reaction)
Annealing; 50℃(10 cycle) → 55℃( 20 cycle)
Extension; 72℃ / 1min

After PCR, clean up the PCR product (1.1kb) by Qiagen column.

Sequence of the amplified fragment (1.1kb)

**CAGATTGTACTGAGAGTGCA**ccataccacagcttttcaattcaattcatcatttttttttattctttttttgatt
tcggtttctttgaaatttttttgattcggtaatctccgaacagaaggaagaacgaaggaaggagcacagacttagat
tggtatatatacgcatatgtagtgttgaagaaacatgaaattgcccagtattcttaacccaactgcacagaacaaaa
acctgcaggaaacgaagataaatcatgtcgaaagctacatataaggaacgtgctgctactcatcctagtcctgttgc
tgccaagctatttaatatcatgcacgaaaagcaaacaaacttgtgtgcttcattggatgttcgtaccaccaaggaat
tactggagttagttgaagcattaggtcccaaaatttgtttactaaaaacacatgtggatatcttgactgatttttcc
atggagggcacagttaagccgctaaaggcattatccgccaagtacaatttttactcttcgaagacagaaaatttgc
tgacattggtaatacagtcaaattgcagtactctgcgggtgtatacagaatagcagaatgggcagacattacgaatg
cacacggtgtggtgggcccaggtattgttagcggtttgaagcaggcggcagaagaagtaacaaaggaacctagaggc
cttttgatgttagcagaattgtcatgcaagggctccctatctactggagaatatactaagggtactgttgacattgc
gaagagcgacaaagattttgttatcggctttattgctcaaagagacatgggtggaagagatgaaggttacgattggt
tgattatgacacccggtgtgggtttagatgacaagggagacgcattgggtcaacagtatagaaccgtggatgatgtg
gtctctacaggatctgacattattattgttggaagaggactatttgcaaagggaagggatgctaaggtagagggtga
acgttacagaaaagcaggctgggaagcatatttgagaagatgcggccagcaaaactaaaaaactgtattataagtaa
a**TGCATGTATACTAAACTCACA**

Bold letters; primer attachment site

Transformation

1. Mix electrocompetent cells (40ul) and a few ul of PCR amplified URA3 gene, and leave it on ice for a few minutes.
2. Give a single pulse (1500V) in ice-cold 0.2cm-gap cuvette.
3. Add 1ml of ice-cold SOS solution to the cuvette immediately after the pulse.
4. Incubate for 1h at 30 ℃ without shaking.
5. Plate cell suspension onto selective agar* (uracil deficient) containing 1M sorbitol.
6. Incubate the plate at 30℃ for 4-7 days (it takes longer than in case of Trp selection).
7. Transfer colonies onto two different selective agar plates, uracil deficient* and tryptophan deficient, by using toothpick.
8. Incubate the plates for 2 days at 30 ℃.
9. Pick some clones that can grow on both plates as candidates, and inoculate them into selective liquid media* (uracil or tryptophan deficient) for extraction of the mutant plasmid (see above).
10. Transform *E. coli* cells with extracted DNA.

*; Use Yeast Synthetic Drop-out Medium Supplements without Uracil from SIGMA (Y1501) to make selective media.

As alternative, spheroplast transformation (see above) with circular construct and PCR amplified marker gene can also be used.