Genetic Manipulation of *Cryptococcus neoformans*

Kwang-Woo Jung,[1] Kyung-Tae Lee,[2] Yee-Seul So,[2] and Yong-Sun Bahn[2,3]

[1]Research Division for Biotechnology, Korea Atomic Energy Research Institute, Jeongeup, Republic of Korea
[2]Department of Biotechnology, Yonsei University, Seoul, Republic of Korea
[3]Corresponding authors: *ysbahn@yonsei.ac.kr*

Cryptococcus neoformans is an opportunistic fungal pathogen, which causes life-threatening meningoencephalitis in immunocompromised individuals and is responsible for more than 1,000,000 infections and 600,000 deaths annually worldwide. Nevertheless, anti-cryptococcal therapeutic options are limited, mainly because of the similarity between fungal and human cellular structures. Owing to advances in genetic and molecular techniques and bioinformatics in the past decade, *C. neoformans*, belonging to the phylum basidiomycota, is now a major pathogenic fungal model system. In particular, genetic manipulation is the first step in the identification and characterization of the function of genes for understanding the mechanisms underlying the pathogenicity of *C. neoformans*. This unit describes protocols for constructing target gene deletion mutants using double-joint (DJ) PCR, constitutive overexpression strains using the histone H3 gene promoter, and epitope/fluorescence protein-tagged strains in *C. neoformans*. © 2018 by John Wiley & Sons, Inc.

Keywords: biolistic transformation • constitutive overexpression • *Cryptococcus neoformans* • double-joint PCR • protein-tagging system

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INTRODUCTION

Cryptococcus neoformans is a basidiomycete pathogen, causing life-threatening cryptococcosis in immunocompromised individuals worldwide (Price & Perfect, 2011). *Cryptococcus gattii*, which is classified as a separate species, mainly causes pneumonia in both immunocompetent and immunosuppressed population, and occurs in geographically restricted regions (Byrnes, Bartlett, Perfect, & Heitman, 2011). The pathogenic *Cryptococcus* species generates infectious propagules in the natural environment, which are subsequently inhaled through the respiratory tract of a susceptible host. *C. neoformans* initially colonizes the lungs and subsequently disseminates via the bloodstream to infect the central nervous system by crossing the blood-brain barrier (Lin & Heitman, 2006). Throughout the whole infection process, *Cryptococcus* utilizes three major virulence factors: a polysaccharide capsule, a polyphenol melanin complex, and the ability to survive at the mammalian body temperature (Idnurm et al., 2005). The capsule prevents cryptococcal cells from being phagocytosed by the host immune system. Melanin protects cells from host-originated oxidative stress and also acts as an antiphagocytic factor (Idnurm et al., 2005). Thermotolerance is generally accepted as a critical virulence factor for most human fungal pathogens (Liu et al., 2008). Nevertheless, the pathobiological signaling network of *C. neoformans* remains elusive. Therefore,

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diverse genetic manipulation techniques have been developed to functionally characterize genes and thereby understand the underlying pathogenic mechanism of *C. neoformans*.

In this unit, we describe the basic genetic manipulation techniques in *C. neoformans*, including construction of gene disruption cassettes using a split marker (see Basic Protocol 1), introduction of the gene disruption cassette by biolistic transformation (see Basic Protocol 2), genomic DNA preparation and verification of genotype in transformants (see Basic Protocol 3), and construction of constitutive overexpression strains (see Basic Protocol 4) and a protein-tagging system (see Basic Protocol 5).

CAUTION: Cryptococcus neoformans is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines for the use of handling of pathogenic microorganisms.

BASIC PROTOCOL 1

CONSTRUCTION OF GENE DISRUPTION CASSETTES USING SPLIT MARKER/DOUBLE-JOINT PCR

Site-directed mutagenesis of a target gene is the most common method of analyzing the function of a gene in any organism. A gene-deletion cassette with a selectable marker is introduced into the genome of the target organism via homologous recombination. Although *C. neoformans* has an intrinsically lower efficiency of homologous recombination than the nonpathogenic budding model yeast *Saccharomyces cerevisiae* (nearly 100%) (Davidson et al., 2000; Orr-Weaver, Szostak, & Rothstein, 1981), linear gene disruption cassettes with >300-bp flanking regions on each side of the target gene can be introduced into the *C. neoformans* genome by biolistic transformation, followed by homologous recombination (Nelson, Pryor, & Lodge, 2003).

To construct the linear gene disruption cassette containing two flanking regions of a target gene and a dominant selectable marker, the conventional overlapping PCR method has been widely used for biolistic transformation (Davidson et al., 2002). More recently, a split marker/double-joint PCR (DJ-PCR)-based method was developed for increasing the efficiency of overlapping PCR required for constructing gene-disruption cassettes and the targeted-integration frequency (Kim, Kim, Yoon, Lee, & Bahn, 2009).

In the first round of the split marker/DJ-PCR, the 5′- and 3′- flanking regions of a target gene and the 5′- and 3′-split regions of a dominant selectable marker are amplified using specific primer pairs. Next, the 5′- and 3′-regions of the target gene disruption cassette are amplified using DJ-PCR (Figure 1).

Materials

Plasmid containing selectable marker [e.g. nourseothricin acetyltransferase (NAT)-encoding gene (pNAT-STM), aminoglycoside phosphotransferase (neomycin;NEO)/G418 resistance-encoding gene (pJAF1), and hygromycin (HYG) B phosphotransferase-encoding gene (pJAF15); pNAT-STMs are available from Jennifer K. Lodge’ laboratory (Washington University) and pJAF1 and pJAF15 are available from Joseph Heitman’ laboratory (Duke University)]
Primers (10 pM)
PCR reagents:
- 10× PCR buffer
- 2.5 mM dNTP
- ExTaq polymerase)

Cryptococcus genomic DNA
Distilled water
1% agarose gel
1 × TAE (Tris acetate EDTA) buffer

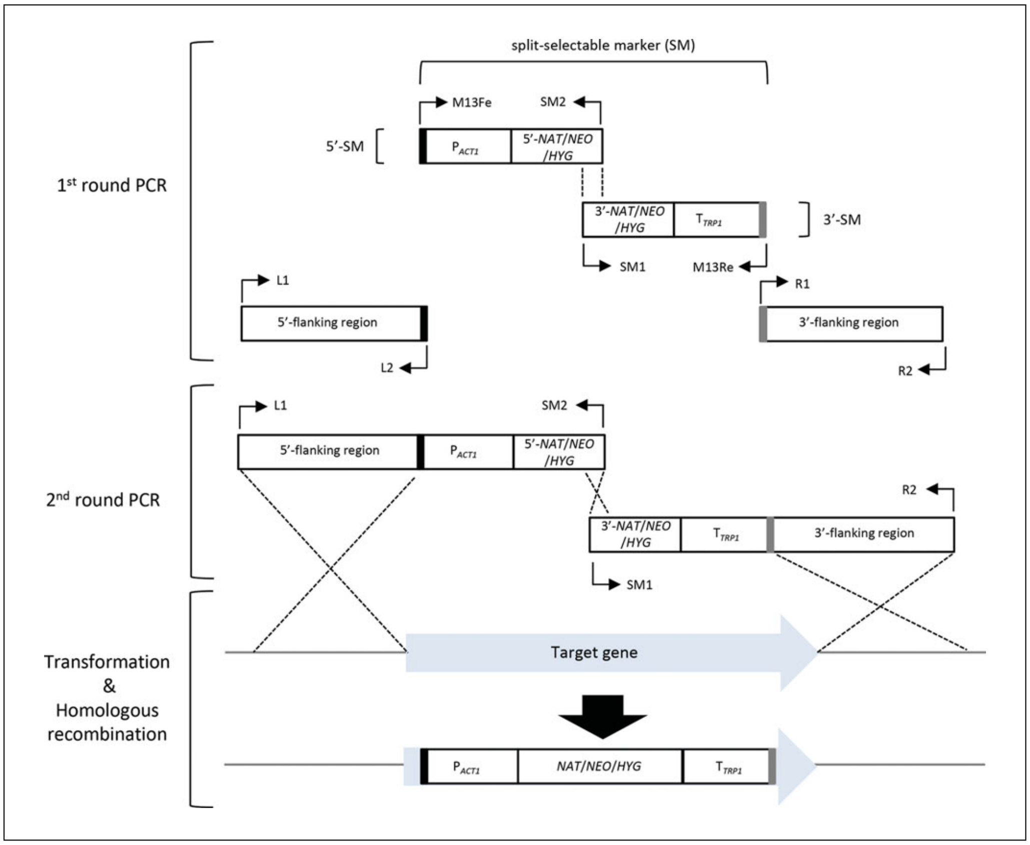

Figure 1 Procedures for targeted gene deletion using the double-joint PCR (DJ-PCR) with split-selectable marker (SM). In the first round of PCR, the 5′- and 3′- flanking regions of the target gene, and the 5′- and 3′-split selectable markers were amplified with the indicated primer pairs, respectively. In the second round of PCR, the 5′- flanking region of the target gene and the 5′-split marker and 3′- flanking region of the target gene and the 3′-split marker were amplified. The two PCR products were combined and introduced into cells using biolistic transformation.

Gel-extraction kit (Cosmo Genetech, cat. no. CMG0112)
Thermal cycler
Sterilized PCR microcentrifuge tubes

Construction of the gene disruption cassette

1. Design and order primer pairs in the 5′- and 3′-flanking homologous regions of a target gene.

   The 5′- and 3′- flanking homologous region of the target gene is ∼0.7- to 1-kb long. The primer pairs for amplification of the 5′- and 3′-flanking homologous regions are designated L1/L2 and R1/R2, respectively. The primer length is 18 to 22 bp. For diagnostic PCR, a screening primer (SO) is located 100 to 200 bp upstream of the L1 primer.

2. Amplify the 5′- and 3′- flanking regions of the target gene with primer pairs L1/L2 and R1/R2, respectively, using the *Cryptococcus* genomic DNA as a template.

   10× ExTaq buffer: 5 μl (final 1× ExTaq buffer)
   2.5 mM dNTP: 4 μl
   Primer L1/L2 (10 pM, for 5′-flanking region of target gene): each 2 μl
   Primer R1/R2 (10 pM, for 3′-flanking region of target gene): each 2 μl
   Template: 1 μg *Cryptococcus* genomic DNA
   ExTaq: 1.25 U
   Distilled water: up to 50 μl.

3. Amplify the 5′-and 3′-split selectable marker genes from plasmids [pNAT-STM for amplification of the *NAT* marker (Nelson, Hua, Pryor, & Lodge, 2001), pJAF1 for amplification of the *NEO* marker (Fraser, Subaran, Nichols, & Heitman, 2003), and pJAF15 for amplification of the *HYG* marker (Fraser et al., 2003)] with primers M13 forward extended (M13Fe) and SM2, and M13 reverse extended (M13Re) and SM1, described in the Table 1, respectively.

   10× ExTaq buffer: 5 µl (final 1× ExTaq buffer)
   2.5 mM dNTP: 4 µl
   Primer M13Fe/SM2 (10 pM, for 5′-split selectable marker gene): 2 µl each
   Primer M13Re/SM1 (10 pM, for 3′-split selectable marker gene): 2 µl each
   Template: 400-500 ng pNAT-STM, pJAF1, or pJAF15
   ExTaq: 1.25 U
   Distilled water: up to 50 µl.

4. Set up the first round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	90 sec	72°C (extension)
1 cycle:	10 min	72°C (final extension).

5. Analyze 2 µl of each PCR product on a 1% agarose gel.

6. After confirming the expected size of the PCR products, recover the agarose gel pieces containing the 5′-flanking region of the target gene and the 5′-split selectable marker (5′-SM) and put them into a sterile 1.5-ml microcentrifuge tube (combined template 1). Separately recover the agarose gel pieces containing the 3′-flanking region of the target gene and the 3′-split selectable marker (3′-SM) and put them into a sterile 1.5-ml microcentrifuge tube (combined template 2).

7. Purify the PCR products using a gel extraction kit.

8. DJ-PCR amplify the 5′-split gene disruption cassette using the combined template 1 (see step 6) and a primer pair L1/SM2, and the 3′-split gene disruption cassette using the combined template 2 (see step 6) and a primer pair R2/SM1 (Figure 1).

   10× ExTaq buffer: 5 µl (final 1× ExTaq buffer)
   2.5 mM dNTP: 4 µl
   Primer L1/SM2 (10 pM, for 5′-split gene disruption cassette): 2 µl each
   Primer R2/SM1 (10 pM, for 3′-split gene disruption cassette): 2 µl each
   Template: 1 µg of each combined first PCR product
   ExTaq: 1.25 U
   Distilled water: up to 50 µl.

9. Set up the second round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	150 sec	72°C (extension)
1 cycle:	10 min	72°C (final extension).

10. Analyze 2 µl of each PCR product on a 1% agarose gel.

11. Combine the two products of DJ-PCR and purify them in the same tube using a gel extraction kit.

Table 1 Primers Used for Genetic Manipulation in *C. neoformans*

Primer Name	Sequence	Comment
M13Fe	GTAAAACGACGGCCAG TGAGC	Primer for dominant selectable marker gene (*NAT, NEO, HYG*)
M13Re	CAGGAAACAGCTATGA CCATG	Primer for dominant selectable marker gene (*NAT, NEO, HYG*)
B1886	TGGAAGAGATGGAT GTGC	Primer for 5′-split-region of *NEO* (Split marker primer 1 for *NEO*, SM1 for *NEO*)
B1887	ATTGTCTGTTGTGCCCAG	Primer for 3′-split-region of *NEO* (Split marker primer 2 for *NEO*, SM2 for *NEO*)
B1454	AAGGTGTTCCCCGACG ACGAATCG	Primer for 5′-split-region of *NAT* (Split marker primer 1 for *NAT*, SM1 for *NAT*)
B1455	AACTCCGTCGCGAGCC CCATCAAC	Primer for 3′-split-region of *NAT* (Split marker primer 2 for *NAT*, SM2 for *NAT*)
B5751	CGAAGAATCTCGTGCTTTC	Primer for 5′-split-region of *HYG* (Split marker primer 1 for *HYG*, SM1 for *HYG*)
B5752	ATTGACCGATTCCTTGCG	Primer for 3′-split-region of *HYG* (Split marker primer 2 for *HYG*, SM2 for *HYG*)
B79	TGTGGATGCTGGCGGAGGATA	Primer in the *ACT1* promoter for diagnostic PCR, pair with SO primer
J12579	TTCCCACCCTCAGCAACGCC	Primer in the *TRP1* terminator for diagnostic PCR, pair with SO primer
B4017	GCATGCAGGATTCGAGTG	Primer for 5′-flanking region of pNEO-H3 and pNAT-H3
B4018	GTGATAGATGTGTTGTGGTG	Primer for 3′-flanking region of pNEO-H3 and pNAT-H3
B5423	GCGGCCGCAGGTGGC GGTGGCTCTGTGAGC AAGGGCGAGGAGCT	Primer for 5′-flanking region of pNEO-GFPht and pHYG-GFPht, pairs with B354
B354	GCATGCAGGATTCGAGTG	Primer for 3′-flanking region of pNEO-GFPht and pHYG-GFPht, pairs with B5423
B5423	GCGGCCGCAGGTG GCGGTGGCTCTGTGA GCAAGGGCGAGGAGCT	Primer for 5′-flanking region of pNAT-GFPht, pairs with B1966
B1966	GGAGCCATGAAGATCCTGA	Primer for 3′-flanking region of pNAT-GFPht, pairs with B5423
B5484	GCGGCCGCAGGTGGCG GTGGCTCTGTGAGC AAGGGCGAGGAGGA	Primer for 5′-flanking region of pNAT-mCherryht, pairs with B354
B354	GCATGCAGGATTCGAGTG	Primer for 3′-flanking region of pNAT-mCherryht, pairs with B5484

continued

Table 1 Primers Used for Genetic Manipulation in *C. neoformans*, *continued*

Primer Name	Sequence	Comment
B5484	GCGGCCGCAGGTGGCGGTGG CTCTGTGAGCAAGGGCGA GGAGGA	Primer for 5′-flanking region of pNEO-mCherryht and pHYG-mCherryht, pairs with B1966
B1966	GGAGCCATGAAGATCCTGA	Primer for 3′-flanking region of pNEO-mCherryht and pHYG-mCherryht, pairs with B5484
B5536	GCGGCCGCAGCATGCGG CGCGCCAGAT	Primer for 5′-flanking region of pNAT-4×FLAGht, pairs with B1966
B1966	GGAGCCATGAAGATCCTGA	Primer for 3′-flanking region of pNAT-4×FLAGht, pairs with B5536
B5536	GCGGCCGCAGCATGCGG CGCGCCAGAT	Primer for 5′-flanking region of pNEO-4×FLAGht and pHYG-4×FLAGht, pairs with B354
B354	GCATGCAGGATTCGAGTG	Primer for 3′-flanking region of pNEO-4×FLAGht and pHYG-4×FLAGht, pairs with B5536
B5534	GCGGCCGCAGCATGCGGC GCGCCATAC	Primer for 5′-flanking region of pNAT-6×HAht, pNEO-6×HAht and pHYG-6×HAht, pairs with B354
B354	GCATGCAGGATTCGAGTG	Primer for 5′-flanking region of pNAT-6×HAht, pNEO-6×HAht and pHYG-6×HAht, pairs with B5534

BASIC PROTOCOL 2

BIOLISTIC TRANSFORMATION

Transformation efficiency in fungi depends not only on the frequency of homologous recombination in a strain, but also on gene delivery methods. Electroporation was the first method used to transfer a cloned gene in *C. neoformans* (Edman & Kwon-Chung, 1990). However, this method is characterized by low transformation efficiency and generation of unstable transformants (Edman, 1992; Edman & Kwon-Chung, 1990; Varma, Edman, & Kwon-Chung, 1992).

Biolistic transformation is an alternative gene-delivery method, in which exogenous DNAs coated on micro-sized metals such as gold are introduced into cells. Although this method was originally developed for transformation of plant cells to overcome the limitation associated with DNA delivery through the thick plant cell wall, it has also been applied to diverse fungal systems (Armaleo et al., 1990; Klein et al., 1987). In *C. neoformans*, biolistic transformation shows a relatively higher efficiency for transferring exogenous DNA into cells and produces more stable transformants than electroporation (Davidson et al., 2000; Toffaletti, Rude, Johnston, Durack, & Perfect, 1993). In this unit, we describe the procedure for biolistic transformation with gene deletion cassettes generated by DJ-PCR, followed by the protocol for diagnostic PCR and Southern hybridization for initial screening of positive transformants.

Materials

C. neoformans
YPD (yeast extract-peptone-dextrose) liquid medium (see recipe)
Distilled water
Solid YPD medium containing 1 M sorbitol (YPD + 1 M sorbitol)
Sterilized 4-mm glass beads
Purified gene-deletion cassettes (see Basic Protocol 1)

100% and 70% ethanol
2.5 M $CaCl_2$ solution
1 M spermidine for molecular biology, minimum 98% GC (Sigma-Aldrich. cat. no. S0266-1G)
100% isopropanol
Solid YPD medium containing antibiotics (100 µg/ml nourseothricin, 50 µg/ml G418, or 150 µg/ml hygromycin B)
TENTS buffer (see recipe)
Phenol:chloroform:isoamyl alcohol (25:24:1) saturated with 10 mM Tris·Cl, pH 8.0, 1 mM EDTA (Sigma-Aldrich, cat. no. P2069-100 ml)
3 M sodium acetate (pH 5.2)
PCR reagents including:
10×PCR buffer
2.5 mM dNTP
ExTaq polymerase
Primer SO (10 pM) and B79 (10 pM)
1% agarose gel
Nourseothricin sulfate (Gold Biotechnology, cat. no. N-500-1)
G418 disulfate salt, powder, cell culture tested (Sigma-Aldrich, cat. no. A1720-5G)
Hygromycin B (Gold Biotechnology, cat. no. H-270-1)

Incubator
Centrifuge
Sterile microcentrifuge tubes
Gold bead microcarrier (Bio-Rad, cat. no. 165-2262)
Macrocarrier membrane (Bio-Rad, cat. no. 165-2335)
Vortex mixer
Rupture disc (Bio-Rad, cat. no. 165-2330/1350 psi)
Stopping screen (Bio-Rad, cat. no. 165-2336)
Biolistic PDS-1000/He particle delivery system (Bio-Rad, cat. no. 165-2257)
Cell scraper
Acid-washed glass-beads, 425 to 600 µm (Sigma-Aldrich, cat. no. G8772-500g) *or* 0.5-mm diameter zirconia/silica (BioSpec Products, cat. no. 11079105z)
Sterile loops *or* toothpicks
Screw cap tubes (USA Scientific, cat. no. 1420-8700)
Bead-beater (BioSpec Products, cat. no. 607)
−80°C freezer
Thermal cycler

Biolistic transformation

1. Incubate *C. neoformans* strains in 50 ml YPD liquid medium for 16 hr at 30°C with shaking (220 to 250 rpm).
2. Next day, spin the cells for 5 min at 1900 × *g*, room temperature, in the benchtop centrifuge.
3. Discard the supernatant and resuspend the cells in 5 ml sterile water.
4. Spread 300 µl of cells per solid-agar plate (YPD + 1 M sorbitol) using 4-mm glass beads.
5. Allow the plates to dry completely on the clean bench and further incubate them for 3 hr at 30°C.
6. During incubation, prepare gene-deletion cassettes with microcarrier according to the following steps 7 to 13.

7. Add 3 to 5 µg purified gene-deletion cassettes in a sterile 1.5-ml microcentrifuge tube.

8. Prepare gold microcarrier particles (0.6-µm gold beads) as follows.

   a. Wash 250 mg of 0.6-µm beads are washed with 1 ml dH_2O and then with 100% ethanol.
   b. Split 1 ml gold beads into four aliquots of 250 µl each.
   c. Add 750 µl of 100% ethanol are added into each aliquot.
   d. Store the microcarrier particle at 4°C until further use.

9. Add 10 µl microcarrier particles, 10 µl of 2.5 M $CaCl_2$, and 1 M spermidine-free base in the same microcentrifuge tube.

10. Vortex the tube for few seconds to ensure uniform coating of the particle with DNA products and let it stand for 5 min.

11. Centrifuge for 1 min at 1100 × *g*, 4°C, and remove the supernatant.

12. Resuspend in 500 µl of 100% ethanol for washing the gold-coated DNA sample.

13. Centrifuge for 1 min at 1100 × *g*, 4°C, and remove the supernatant.

14. Add 12 µl of 100% ethanol into the tube and resuspend the particles by pipetting.

15. Wash a macrocarrier membrane and the stopping disc with 100% ethanol. Wash the rupture disc (1350 psi rupture disc) with isopropanol. Dry the macrocarrier membrane, stopping disc, and rupture disc on the clean bench.

16. Place the macrocarrier membrane using the red plastic cylindrical holder on the macrocarrier holders.

17. Transfer the gold-coated DNA sample onto the center of the macrocarrier membrane and dry the disc until ethanol is evaporated.

18. Perform transformation with biolistic PDS-1000/He particle delivery system as follows:

   a. Before transformation, sanitize all internal parts of the device with 70% ethanol.
   b. Turn on the pump and open the valve of a helium tank.
   c. Turn the knob of the helium tank clockwise until pressure reaches ~2000 psi.
   d. Place the rupture disc into the holder hanging from the ceiling of the chamber and screw the holder back.
   e. Place the stopping screen at the bottom of the disc chamber.
   f. Set the macrocarrier holder with gold-coated DNA side-down into the disc chamber.
   g. Screw the silver cap and load the disc chamber in the top slot of the chamber.
   h. Remove the lid of plate that was incubated for 3 hr at 30°C and load it in the second slot from the top of the chamber.
   i. Close the door of the biolistic transformation machine and turn it on.
   j. Push the "*vacuum*" button until the pressure gauge reaches ~28.5 inches of Hg.
   k. Switch quickly to the "*hold*" button and then hold down the "*fire*" button until you hear the rupture disc break.
   l. Release the "*fire*" button and then push the "*vent*" button to release the vacuum from the chamber.

19. Place the transformed plates with the lid on in a 30°C incubator and further incubate them for 4 hr to allow for recovery.

20. After 4 hr incubation, add 1 ml YPD liquid medium on the transformed plates, recover transformed cells using a cell scraper, and transfer them on the agar-based

selection medium containing nourseothricin (100 μg/ml), G418 (50 μg/ml), or hygromycin B (150 μg/ml).

21. Spread the cells on the plate using sterilized glass beads and dry the plate completely on the clean bench.

22. Incubate the plates in a 30°C incubator until transformants appear (for 3 to 7 days).

Modified "smash & grab" genomic DNA preparation method for diagnostic PCR

23. Transfer transformants from the selective medium plates using a sterile loop or toothpick on a new agar-based selective plate with grids to make a master plate and incubate it for 1 to 2 days at 30°C.

24. Pick transformants grown on the master plate and transfer a portion of it into 2 ml YPD liquid medium for 16 hr at 30°C with shaking (220 to 250 rpm).

25. Centrifuge the cells for 5 min at 1900 × *g*, room temperature, in the benchtop centrifuge and remove the supernatant.

26. Resuspend the cells in 0.5 ml TENTS buffer and transfer them into 2-ml screw-cap tubes containing 0.2 ml of acid-washed glass-bead or 0.5 mm-diameter zirconia/silica.

27. Add 0.5 ml phenol-chloroform-isoamyl alcohol (25:24:1) to the screw-cap tubes.

28. Disrupt the cells for 1 to 2 min using a bead-beater.

29. Centrifuge the screw-cap tubes for 5 min at 11,400 × *g*, 4°C, to separate the organic and aqueous phases and transfer the aqueous supernatant (approximately 450 μl) into a new 1.5-ml microcentrifuge tube.

    After centrifugation, the white layer appears between organic (bottom) and aqueous (upper) phase.

30. Add 50 μl of 3 M sodium acetate (pH 5.2) and 1 ml of 100% cold ethanol into the microcentrifuge tube containing the supernatant to the precipitate DNA and invert it several times.

31. Place the DNA sample for 30 min at −80°C.

    At this step, the DNA sample can be stored at −80˚C until further use.

32. Centrifuge the tubes for 20 min at 11,400 × *g*, 4°C to precipitate DNA and discard the supernatant.

33. Add 0.5 ml of 70% ethanol into the precipitated DNA for washing.

34. Centrifuge the tubes for 5 min at 11,400 × *g*, 4°C, and discard the supernatant.

35. Air-dry the DNA pellets and dissolve it in 50 μl distilled water.

36. Store it at −20°C until further use.

37. Mix the following ingredients well in a sterile 0.5-ml microcentrifuge tube.

    The B79 primer that binds to the ACT1 promoter region of NAT, NEO, or HYG markers.

    10× ExTaq buffer: 2.5 μl (final 1 × ExTaq buffer)
    2.5 mM dNTP: 2 μl
    Primer SO (10 pM): 2 μl
    Primer B79 (10 pM): 2 μl
    Template: 1 μg of extracted genomic DNA from "smash and grab" mini-preparation method

ExTaq: 1 U
Distilled water: up to 25 µl.

38. Set up the diagnostic PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
25 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	90 sec	72°C (extension)
1 cycle:	10 min	72°C (final extension).

39. Analyze 2-µl PCR products on a 1% agarose gel to screen for positive clones.

BASIC PROTOCOL 3

VERIFICATION OF *C. neoformans* MUTANT GENOTYPE BY SOUTHERN BLOTTING

The transferred DNAs can be additionally integrated at an unrelated genomic locus via ectopic integration, which can result in unexpected phenotypic change. However, the diagnostic PCR allows us to confirm only targeted integration of the transferred DNA, but not ectopic integration of gene-disruption cassettes at non-targeted genomic loci. Therefore, after initial screening of positive transformants by diagnostic PCR, Southern blot analysis is widely used to verify the genotype of a constructed mutant (Southern, 1975).

For Southern blot analysis, high-quality genomic DNA should be prepared first and digested with a restriction enzyme to obtain useful genotypic information. The restriction digested genomic DNA is then separated by agarose gel electrophoresis, denatured using an alkaline solution, and immobilized on nitrocellulose or nylon membrane. Then, the membrane is hybridized with a radio- or non-radiolabeled gene-specific probe, washed, and developed on an X-ray film.

Isolation of high-quality genomic DNA is challenging because of the rigidity of the fungal cell wall. Hence, several techniques have been used to circumvent this problem (Cardinali, Liti, & Martini, 2000; Holm, Meeks-Wagner, Fangman, & Botstein, 1986; Varma & Kwon-Chung, 1991). In particular, cetyl trimethyl ammonium bromide (CTAB) extraction buffer is widely used for extraction of high-quality genomic DNA of *C. neoformans* (Doyle & Doyle, 1987). In this unit, we describe methods for genomic DNA isolation of *C. neoformans* using the CTAB extraction buffer and Southern blot analysis.

Materials

Cyptococcus cells
YPD liquid medium
−80°C freezer
Phosphate-buffered saline (PBS)
Liquid nitrogen
CTAB extraction buffer (see recipe)
Chloroform (Junsei, cat. no. 28560-0350)
Isopropanol (Junsei, cat. no. 64605-0380)
70% ethanol
TE buffer
RNase A (10 µg/ml)
Distilled water
Restriction enzymes
1% agarose gel (1×TAE buffer)
Denaturation buffer (see recipe)

Neutralization buffer (see recipe)
20× SSC (Saline-sodium citrate) solution (see recipe)
5× SSC solution
10× SSC solution
Modified Church hybridization solution (see recipe)
Radioisotope labeling agents: Klenow enzyme buffer, random hexamer primer (Roche, cat. no.11277081001) dNTP without dCTP, and Klenow enzyme)
α-[^{32}P] dCTP (Perkin Elmer Life Sciences, cat. no. NEG-513H)
Clean up kit (GE Healthcare, ct. no. 27512001)
Washing solution I (see recipe)
Washing solution II (see recipe)

Incubator
50- and 15-ml conical tubes
Centrifuge
Lyophilizer
3-mm glass beads
Vortex mixer
Water bath
Nylon membrane (Millipore, cat. no. INYC00010)
Solid support
Plastic dish
Whatman paper
Plastic wrap
Pencils
UV cross-linker (1200 J/m^2 UV exposure)
Autoradiography film
X-ray Developer (Vivid) and fixer (Vivid)

Preparation of Cryptococcus genomic DNA using CTAB extraction buffer

1. Culture cells in 50 ml YPD liquid medium for 1 to 2 days with 200 to 220 rpm at 30°C.
2. Transfer the cells into a 50-ml conical tube and centrifuge for 5 min at 1900 × *g*, room temperature.
3. Discard the supernatant and add 20 ml PBS buffer to the falcon tube for washing.
4. Centrifuge the tube and discard the supernatant.
5. Freeze the precipitated cells in liquid nitrogen for 3 to 4 hr and then lyophilize the samples for 16 hr.
6. Add approximately 3 to 5 ml of 3-mm glass beads into the lyophilized cell pellet and shake or vortex it vigorously until a fine powder is generated.
7. Add 10 ml CTAB extraction buffer and mix by vortexing.
8. Incubate the samples in a water chamber for 30 min at 65°C and then cool under cold tap water for 10 min.
9. Add 10 ml chloroform and mix by inverting 10 to 20 times in a chemical hood.
10. Centrifuge the samples for 10 min at 1900 × *g*, 4°C to separate organic and aqueous phases.
11. Transfer 7 ml of the aqueous phase (supernatant) into a new 15-ml conical tube containing equal volume of isopropanol (7 ml).

12. Immediately mix by inversion for 10 to 20 times and incubate for 10 min.

    Thread-like structure/precipitate will appear in this step. If the event does not occur, incubate for longer time.

13. Centrifuge the sample for 5 min at 1900 × *g*, 4°C and then discard the supernatant.
14. Add 5 ml of 70% ethanol into the DNA pellet for washing and centrifuge for 5 min at 1900 × *g*, 4°C.
15. Discard the supernatant and then dry the DNA pellet in a chemical hood for 30 min.
16. Resuspend the DNA pellet with 200 to 500 µl TE buffer and dissolve it completely in a water chamber at 65°C.
17. Add 2 µl RNase A to remove any ribosomal RNA and incubate it for 1 to 2 hr at 37°C.

Southern blot analysis

This step involves digestion of the isolated genomic DNA with restriction enzymes appropriate for checking the targeted gene deletion. The digested genomic DNAs are separated on 1% agarose gel. After gel electrophoresis, the digested genomic DNAs are denatured, neutralized, and transferred onto nitrocellulose or nylon membranes by capillary transfer. After transferring DNA, the membrane is exposed to UV irradiation to immobilize the DNA. The gene-specific and radiolabeled probe is hybridized with the denatured genomic DNA on the membrane.

18. Set up the genomic DNA digestion reaction as follows:

    10× restriction enzyme buffer: 5 µl (final 1 × ExTaq buffer)
    Genomic DNA: 20 µl (total 50 to 100 µg genomic DNA)
    Restriction enzyme: 10 to 20 U
    Distilled water: up to 50 µl.

19. Digest genomic DNA for 16 hr with appropriate restriction enzymes at the indicated temperature recommended by manufacturer.
20. Run the digested genomic DNA on a 1% agarose gel.

    Maintain a low voltage through the gel (about <10 V/cm) to allow slow migration of DNA. Mark the size marker on the gel with knife or pencil before gel denaturation.

21. Denature the agarose gel in the denaturation buffer for 45 min with gentle shaking.
22. Discard the denaturation buffer and rinse the gel with distilled water.
23. Cover the gel with neutralization buffer for 45 min with gentle shaking.
24. Discard the neutralization buffer and rinse the gel with distilled water.
25. Transfer the digested genomic DNA to the nylon membrane in 10× SSC buffer via capillary blotting for 16 hr (Figure 2).

    a. Place a solid support, which is larger than the gel, and pour 10× SSC buffer in a plastic dish.
    b. Cover the support with the Whatman 3 MM filter paper and wet the paper with 10× SSC buffer.
    c. Put the gel on the filter paper upside down.
    d. Place plastic wrap over the edge of the gel.
    e. Place a nylon membrane, which is a slightly bigger size than the exposed surface of gel and submerged in the distilled water for 1-2 min just before using, on the exposed surface of gel.

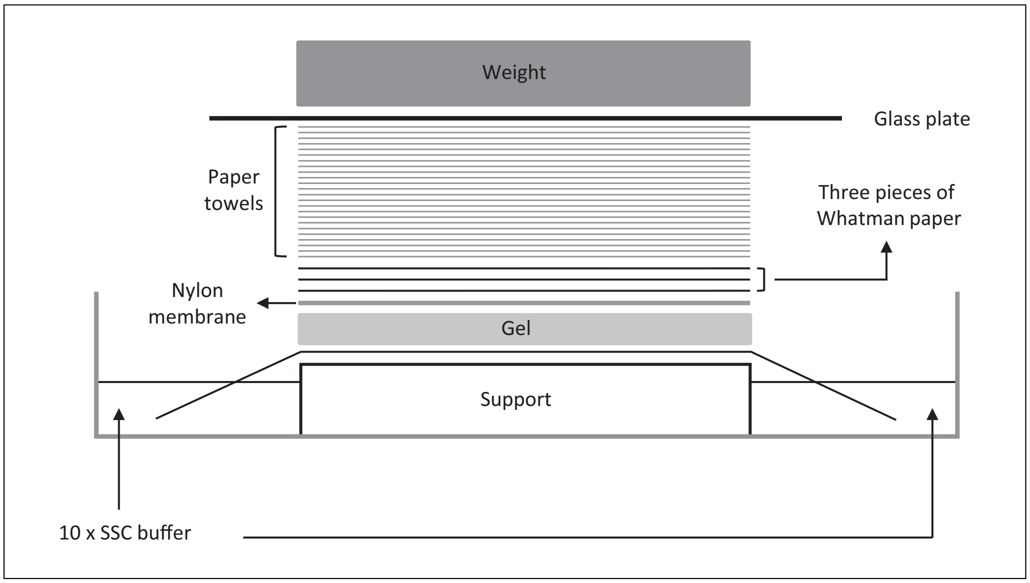

Figure 2 Capillary transfer of restriction enzyme-digested DNA to membrane. 10× SSC transfer buffer transfers the restriction enzyme-digested DNA from the gel to the membrane by capillary action. The negative charge of the DNA binds to the positive charge of the membrane through ion exchange interaction.

f. Place the plastic wrap over the edge of the nylon membrane.
g. Put three pieces of the Whatman 3 MM filter paper to the same size as the nylon membrane.
h. Stack paper towels on the Whatman 3MM filter paper to a height of 15 cm.
i. Lay a glass plate or something as flat on the paper towels and then place weight on top to induce capillary transfer.

26. Remove the paper towel stacks from the transfer and mark the side of the membrane with a pencil to check the DNA size marker.

27. Fix the transferred nucleic acids on the membrane by exposure to 1200 J/m^2 ultraviolet (UV) radiation in an UV cross-linker.

Store the membrane in aluminum foil until it is used.

28. Roll the membrane inside the hybridization tube.

Add a small amount of 5× SSC to the tube or wet the membrane in 5× SSC buffer to easily roll the membrane in the tube. Place the membrane on the inner side of the hybridization tube to expose it to the hybridization buffer.

29. Pre-hybridize the membrane for 1 to 3 hr at 65°C with 10 ml modified Church hybridization buffer in the hybridization tube with a rotating chamber.

Mix the hybridization buffer thoroughly.

30. During pre-hybridization, radiolabel the gene-specific probes by random priming as follows:

a. Denature gene-specific probe (5 µl PCR product, approximately 2 µg) in the microcentrifuge tube at 95°C for 5 min.
b. Cool the denatured probe on ice for 5 min.
c. To label the probe with radioisotope, add the following reagents (2 µl of 10×Klenow buffer, 2 µl random hexamer, 9 µl of 5 mM dNTP mixture without dCTP, 2 µl α-[^{32}P] dCTP, and 1 µl Klenow enzyme).
d. Incubate the reaction for 45 min at 37°C.

e. After incubation, clean the radiolabeled probe using the clean-up kit.
f. Denature the radiolabeled probe for 5 min at 95°C, followed by cooling of the denatured probe.

31. Add the denatured and radiolabeled probe to the hybridization tube and then hybridize for 12 to 24 hr at 65°C.

Avoid placing the radiolabeled probe directly on the membrane and bubble formation during hybridization.

32. After hybridization, wash the membrane twice for 15 min with washing solution I and solution II.

33. Expose the membrane to an autoradiography film and develop it overnight.

Cover the membrane with a wrap and do not expose directly to the autoradiography film.

BASIC PROTOCOL 4

CONSTRUCTION OF STRAINS CONSTITUTIVELY OVEREXPRESSING A TARGET GENE

If a gene of interest is essential for viability, its function cannot be characterized using a deletion strain. In such cases, strains overexpressing the target gene can be used for predicting its function. Here, we describe a method for generating strains overexpressing the target gene by inserting the histone H3 gene promoter with dominant selectable marker in the promoter region of the target gene.

Materials

Primers (10 pM)
PCR reagents including
- 10× PCR buffer
- 2.5 mM dNTP
- ExTaq polymerase

Cryptococcus genomic DNA
Distilled water
Plasmid containing the H3 gene promoter and a selection marker [e.g., pNAT-H3 or pNEO-H3; pNAT-H3 and pNEO-H3 are available from Yong-Sun Bahn's laboratory (Yonsei University)]
1% agarose gel
PCR purification or gel extraction kit
Sterilized PCR microcentrifuge tubes

Construction of gene cassette to replace the native promoter with the histone H3 promoter

1. Design primer pairs in the 5′-flanking promoter region and 3′-flanking exon region (the first exon region) of the target gene.

The 5′-flanking region and 5′-exon region of the target gene are of approximately 0.7 to 1 kb. The 5′-exon region starts from +1 (ATG) to +700 to +1000. The primer pairs for amplification of the 5′-flanking promoter region and the 3′-flanking exon region are designated OE-L1/OE-L2 and OE-R1/OE-R2, respectively. The primer length is about 18 to 22 bp. For diagnostic PCR, a screening primer (OE-SO) is located 100~200 bp upstream of the OE-L1 primer.

2. Amplify the 5′-flanking region and the 3′-flanking region of the target gene with primer pairs OE-L1/OE-L2 and OE-R1/OE-R2, respectively, using genomic DNA as the template in the first round of PCR (Figure 3).

10× ExTaq buffer: 5 µl (final 1× ExTaq buffer)

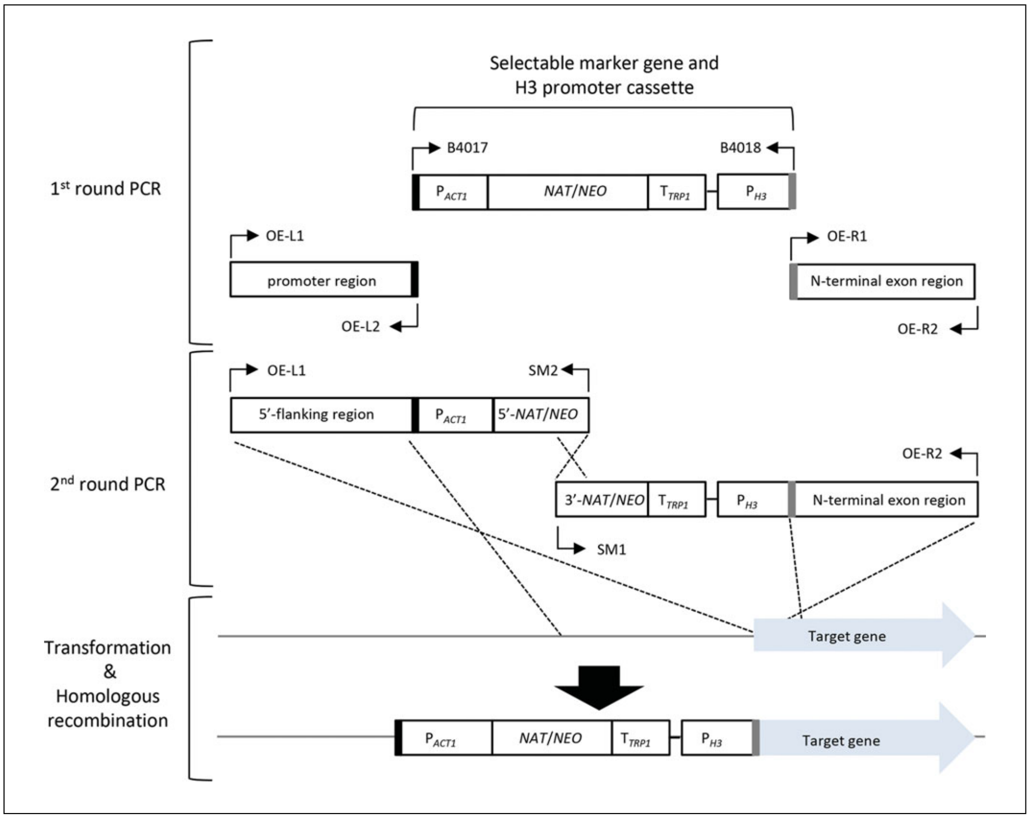

Figure 3 Procedures for insertion of the constitutive overexpression promoter by double-joint PCR (DJ-PCR) with *NAT*/*NEO*-split marker. In the first round of PCR, the promoter region and the N-terminal exon region of the target gene, the *NAT*/*NEO* marker, including the H3 promoter were amplified as illustrated. In the second round of PCR, the promoter region of the target gene and the 5′-*NAT*/*NEO*-split marker were amplified with the indicated primer pairs OE-L1/SM2. Similarly, the N-terminal exon region of the target gene and the 3′- *NAT*/*NEO*-split marker were amplified using primer pairs OE-R2/SM1. The two PCR products were combined and introduced into cells via biolistic transformation.

2.5 mM dNTP: 4 µl
Primer OE-L1/OE-R1 (10 pM): 2 µl each
Primer OE-L2/OE-R2 (10 pM): 2 µl each
Template: 1 µg *Cryptococcus* genomic DNA
ExTaq: 1.25 U
Distilled water: up to 50 µl.

3. Amplify the selection marker-H3 promoter region using pNEO-H3 (a plasmid containing the *NEO* marker and the H3 promoter) or pNAT-H3 (a plasmid containing the *NAT* marker and the H3 promoter) as the template with the primer pair B4017/B4018 (Figure 3).

Amplify two independent sets of selectable markers and H3 promoter from the plasmid.

10× ExTaq buffer: 5 µl (final 1 × ExTaq buffer)
2.5 mM dNTP: 4 µl
Primer B4017/B4018 (10 pM): 2 µl each
Template: 400 to 500 ng pNEO-H3 (or pNAT-H3)
ExTaq: 1.25 U
Distilled water: up to 50 µl.

4. Set up the first round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	90 sec	72°C (extension for 150 s at 72°C for amplification of the selectable marker gene and H3 promoter)
1 cycle:	10 min	72°C (final extension).

5. Analyze 2 µl PCR products on a 1% agarose gel.
6. For the second round of PCR, combine the PCR products of the 5′-flanking promoter region of the target gene and selection marker-H3 promoter region. Separately combine the PCR products of the 3′-flanking exon region of the target gene and the selection marker-H3 promoter region. Purify the combined PCR products using a PCR purification or gel extraction kit
7. Amplify the 5′- and 3′-split regions of the overexpression gene cassette with primer pairs OE-L1/SM2 and OE-R2/SM1, respectively, using the first-round PCR products as templates in the second round of PCR (Figure 3).

   10× ExTaq buffer: 5 µl (final 1 × ExTaq buffer)
   2.5 mM dNTP: 4 µl
   Primer OE-L1/SM2 (10 pM): 2 µl each
   Primer OE-R2/SM1 (10 pM): 2 µl each
   Template: 1 µg of each combined first round PCR product
   ExTaq: 1.25 U
   Distilled water: up to 50 µl.

8. Set up the second round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	180 sec	72°C (extension)
1 cycle:	10 min	72°C (final extension).

9. Analyze 2 µl PCR products on a 1% agarose gel.
10. Combine the two PCR products and purify them using PCR-purification or gel extraction kit.
11. Introduce the split overexpression gene cassettes into *C. neoformans* by biolistic transformation (see Biolistic transformation section)
12. Perform diagnostic PCR with primer pairs B79/OE-SO to screen for positive transformants.
13. Perform Southern blot analysis to verify the correct genotype of the selected positive transformants (see Basic Protocol 3).
14. After verification of the correct genotype, perform quantitative reverse transcription-PCR (qRT-PCR) or northern blot analysis to confirm overexpression of the target gene.

BASIC PROTOCOL 5

VERSATILE PROTEIN-TAGGING SYSTEMS

Protein tagging systems enable understanding of the biological role of a target protein by providing information on changes in protein expression, protein-protein interactions, and protein subcellular localization. Recently, we constructed 12 different protein-tagging plasmids containing FLAG, HA, mCherry, and GFP with *NAT*, *NEO*, or *HYG* selection

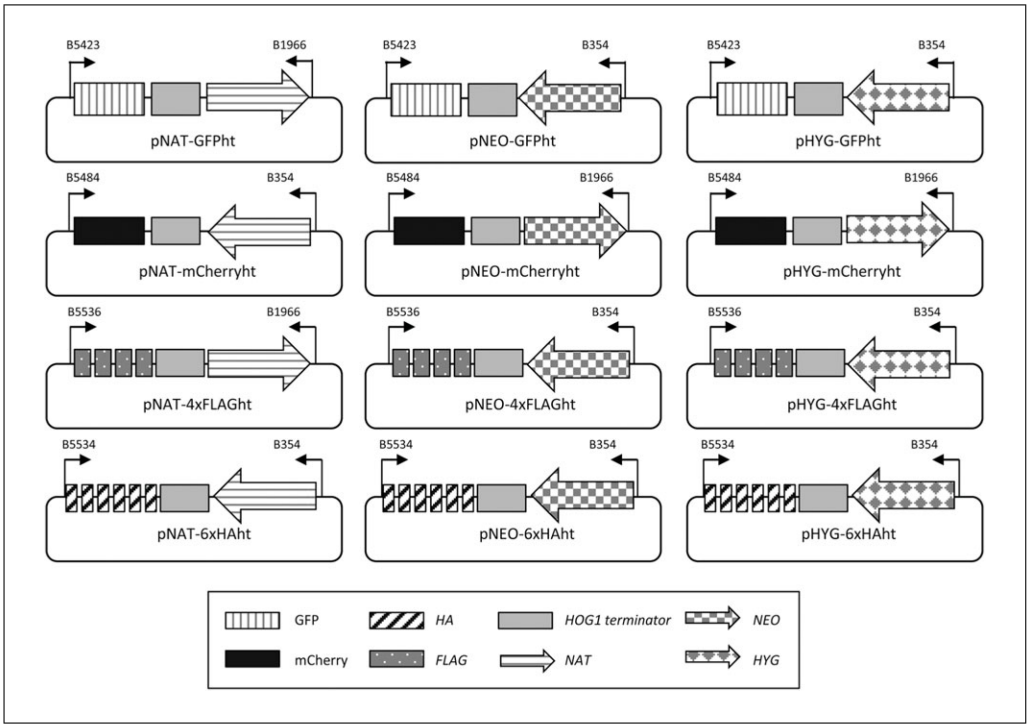

Figure 4 Schematic diagrams of the versatile C-terminal epitope-tagging plasmid set. The C-terminal epitope-tagging plasmid set is composed of 12 plasmids, which include GFP, mCherry, 4× FLAG, and 6× HA epitope with three different selectable dominant markers. The arrows indicate that primers are used for construction of the C-terminal tagging cassette.

markers (Figure 4) (So, Yang, Jung, Huh, & Bahn, 2017). In this protocol, we describe procedures for construction of strains tagged with epitopes (FLAG or HA) or fluorescent proteins (GFP or mCherry) for performing immunoblot assay and co-immunoprecipitation, and studying protein localization using the tagging plasmids.

Materials

Primers (10 pM)
PCR reagents including:
- 10× PCR buffer
- 2.5 mM dNTP
- ExTaq polymerase

Cryptococcus genomic DNA
Distilled water
12 different tagging plasmids (Addgene; www.addgene.org)
1% agarose gel
PCR purification or gel extraction kit
Sterilized PCR microcentrifuge tubes

Construction of strains harboring the C-terminal epitope tag by a split marker/double joint PCR method

1a. Design primer pairs in the 5′- flanking exon region (the last exon region) and 3′-flanking terminator region of a target gene (Figure 5).

The 5′-flanking exon and 3′-flanking terminator regions of the target gene are approximately 0.7 to 1 kb in length. The primer pairs for amplification of the 5′- and 3′-flanking regions are designated "epitope name"-L1/"epitope name"-L2 and "epitope name"-R1/"epitope name"-R2, respectively. The primer length is about 18 ~ 22 bp. The "epitope name"-L2 primer does not contain a "stop codon", which enables generation of a translational fusion between the target gene and the epitope. For diagnostic PCR,

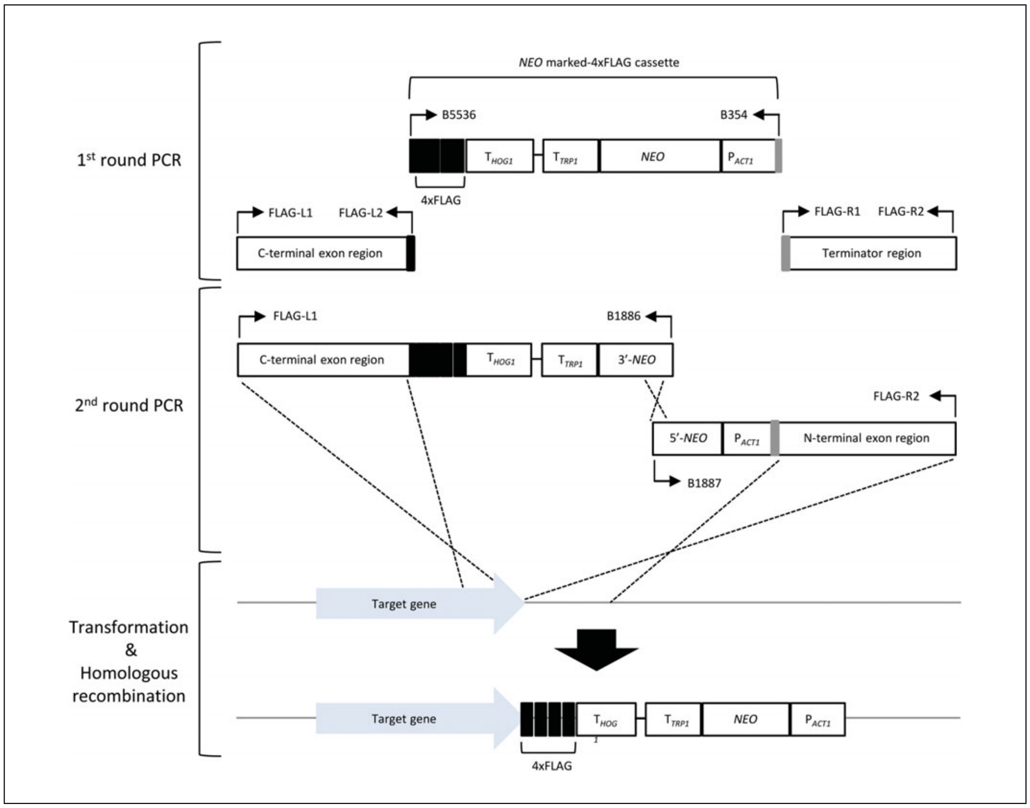

Figure 5 Construction of strains with C-terminal 4× FLAG-tag. In the first round of PCR, the C-terminal exon region and the terminator region of the target gene, and the *NEO* marked-4× FLAG cassettes were amplified. In the second round of PCR, the C-terminal exon region of the target gene and the 3′-*NEO* marked-4× FLAG cassettes were amplified with the primer pairs FLAG-L1/B1886. Similarly, the 5′-*NEO* marked-4× FLAG cassettes and the terminator region of the target gene were amplified using primer pairs FLAG-R2/B1887. Next, the two PCR products were combined and introduced into cells via biolistic transformation.

a screening primer (“epitope name”-SO) is located 100 to 200 bp upstream of the “epitope name”-L1 primer.

2a. Amplify the 5′- flanking exon and 3′- flanking terminator regions of the target gene with primer pairs, “epitope name”-L1/“epitope name”-L2 and “epitope name”-R1/“epitope name”-R2, respectively, using genomic DNA as a template in the first round of PCR (here, we describe procedures with 4× FLAG as the epitope) (Figure 5).

10× ExTaq buffer: 5 µl (final 1 × ExTaq buffer)
2.5 mM dNTP: 4 µl
Primer FLAG-L1/FLAG-L2 (10 pM): 2 µl each
Primer FLAG-R1/FLAG-R2 (10 pM): 2 µl each
Template: 1 µg *Cryptococcus* genomic DNA
ExTaq: 1.25 U
Distilled water: up to 50 µl.

3a. Amplify the selection marker-epitope region from one of the tagging plasmids (Figure 4; here, we use the plasmid pNEO-4× FLAG as template) with primer pairs listed in Table 1 (Figure 5).

Amplify two independent sets of gene-marked epitope from the plasmid.

10× ExTaq buffer: 5 µl (final 1 × ExTaq buffer)
2.5 mM dNTP: 4 µl

Primer B5536/B354 (10 pM): 2 μl each
Template: 400-500 ng of pNEO-4× FLAG
ExTaq: 1.25 U
Distilled water: up to 50 μl.

4a. Set up the first round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	90 sec	72°C (extension for 180 sec at 72°C for amplification of selectable marker gene and epitope)
1 cycle:	10 min	72°C (final extension).

5a. Analyze 2 μl PCR products on a 1% agarose gel to validate the PCR amplification.

6a. For the second round of PCR, combine the PCR products of the 5′-flanking exon region of the target gene and the selection marker-epitope region. Separately combine the PCR products of the 3′-flanking terminator region of the target gene and the selection marker-epitope region. Purify them using the PCR purification or gel extraction kit.

7a. In the second round of PCR, amplify the 5′-and 3′-split regions of the "target gene: 4 × FLAG:NEO" cassette with primer pairs FLAG-L1/B1886 and FLAG-R2/B1887, respectively, using the first-round PCR products as templates (Figure 5).

10× ExTaq buffer: 5 μl (final 1 × ExTaq buffer)
2.5 mM dNTP: 4 μl
Primer FLAG-L1/B1886 (10 pM): 2 μl
Primer FLAG-R2/B1887 (10 pM): 2 μl
Template: 1 μg each of the combined first round PCR product
ExTaq: 1.25 U
Distilled water: up to 50 μl.

8a. Set up the second round of PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	180 sec	72°C (extension)
1 cycle:	10 min	72° (final extension).

9a. Analyze 2 μl PCR products on a 1% agarose gel.

10a. Combine the two DJ-PCR products and purify them using the PCR purification or gel extraction kit.

11a. Introduce the split "target gene:4× FLAG:NEO" cassettes into *C. neoformans* by biolistic transformation (see Basic Protocol 2)

12a. Perform diagnostic PCR using the J12579/FLAG-SO primer pair to screen for positive transformants.

13a. Perform Southern blot analysis to verify the correct genotype of the selected transformants (see Basic Protocol 3).

14a. After genotypic verification, perform immunoblot analysis using anti-epitope antibody to confirm whether a correct translational fusion between the selected epitope and the target gene has occurred, and a phenotypic analysis to determine whether

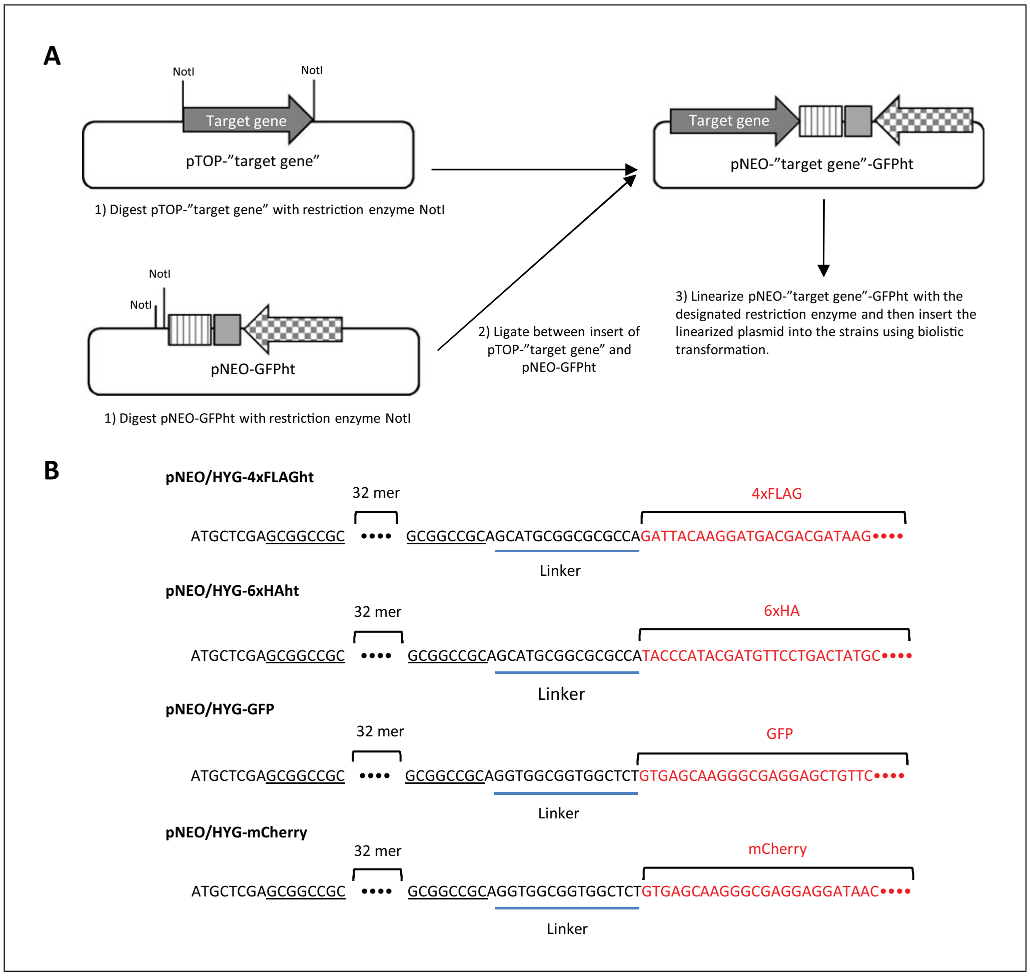

Figure 6 Strategy for construction of strains containing the C-terminal epitope tag by plasmid complementation. (**A**) The procedure for construction of the pNEO-target gene-GFPht. (**B**) Cloning sites in the versatile C-terminal epitope-tagging plasmid sets. The underline indicates the NotI restriction site (5′-GCGGCCGC-3′). The linker provides flexibility between the fused proteins.

the constructed strain is phenotypically similar to the wild-type strain and the functionality of the epitope-tagged protein.

Construction of strains containing the C-terminal epitope tag by plasmid complementation

1b. Design primer pairs to amplify the promoter and open reading frame (ORF) without the stop codon of a target gene for construction of the “target gene”Δ::“target gene-GFP” construct (epitope-tagged complemented strain; here, we use pNEO-GFPht as an example) (Figure 6).

Before designing the primers, check for the presence of NotI restriction enzyme sites in the target gene. If the gene contains a NotI restriction enzyme site, do not use these plasmid sets. Furthermore, because the NAT selection marker contains a NotI site, the use of plasmids containing the NAT selection marker is not appropriate for C-terminal epitope tag by plasmid complementation. To generate a translational fusion between the target gene and an epitope, a primer should be designed without a stop codon of the ORF.

2b. Amplify the promoter and ORF of the “target gene” with the primer pairs, which contain the *Not*I restriction enzyme site.

10× ExTaq buffer: 5 µl (final 1× ExTaq buffer)
2.5 mM dNTP: 4 µl

Primer GFP-1/GFP-2 (10 pM): 2 μl each
Template: 1 μg *C. neoformans* genomic DNA
ExTaq: 1.25 U
Distilled water: up to 50 μl.

3b. Set up the PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	XX	72°C (XX depends on the amplicon size, and is usually calculated from the polymerase's speed of 1 kb/1 min)
1 cycle:	10 min	72°C (final extension).

4b. Perform TA cloning with the PCR product using the pTOP-V2 plasmid to produce the plasmid pTOP-"target gene".

5b. Confirm DNA sequence error(s) of the PCR-amplified insert.

6b. After confirming the DNA sequence, subclone a *Not*I restriction enzyme-digested PCR insert of the pTOP-"target gene" into the pNEO-GFPht plasmid to construct pNEO-"target gene"-GFPht.

Confirm ligation of your gene in the proper orientation by restriction enzyme digestion.

7b. Insert the designated restriction enzyme-digested pNEO-"target gene"-GFPht into the "target gene" mutant using biolistic transformation.

8b. Perform diagnostic PCR with the designated primer pairs to check the targeted or ectopic integration.

9b. After verification of targeted integration, perform phenotypic analysis to check whether all the mutant phenotypes are restored to wild type to confirm the functionality of the tagged protein.

Construction of strains harboring the N-terminal epitope tag by plasmid complementation

1c. Design primer pairs to amplify the promoter (Pro) and ORF without the start codon and terminator (ORFter) of a target gene and the RFP region (RFP) for construction of the "target gene"Δ::"target gene-RFP" (N-terminal epitope-tagged complemented strain; here, we use pNEO-RFPht as an example) (Figure 7A).

In this part of the protocol, we describe a method for construction of pTOP-Pro+RFP+ORFter by cloning PCR products containing the promoter, ORF, and terminator, and the RFP region. Alternatively, you can use overlapping PCR to construct a gene cassette containing the promoter, ORF, and terminator, and the RFP region (Figure 7B).

2c. Amplify the promoter and ORF, and terminator of the "target gene" with the primer pairs using *C. neoformans* genomic DNA as the template (Figure 7A).

10× ExTaq buffer: 5 μl (final 1× ExTaq buffer)
2.5 mM dNTP: 4 μl
Primer Pro_L1/Pro_R1 (10 pM): 2 μl each
Primer ORF_L1/ORF_R1 (10 pM): 2 μl each
Template: 1 μg *C. neoformans* genomic DNA
ExTaq: 1.25 U
Distilled water: up to 50 μl.

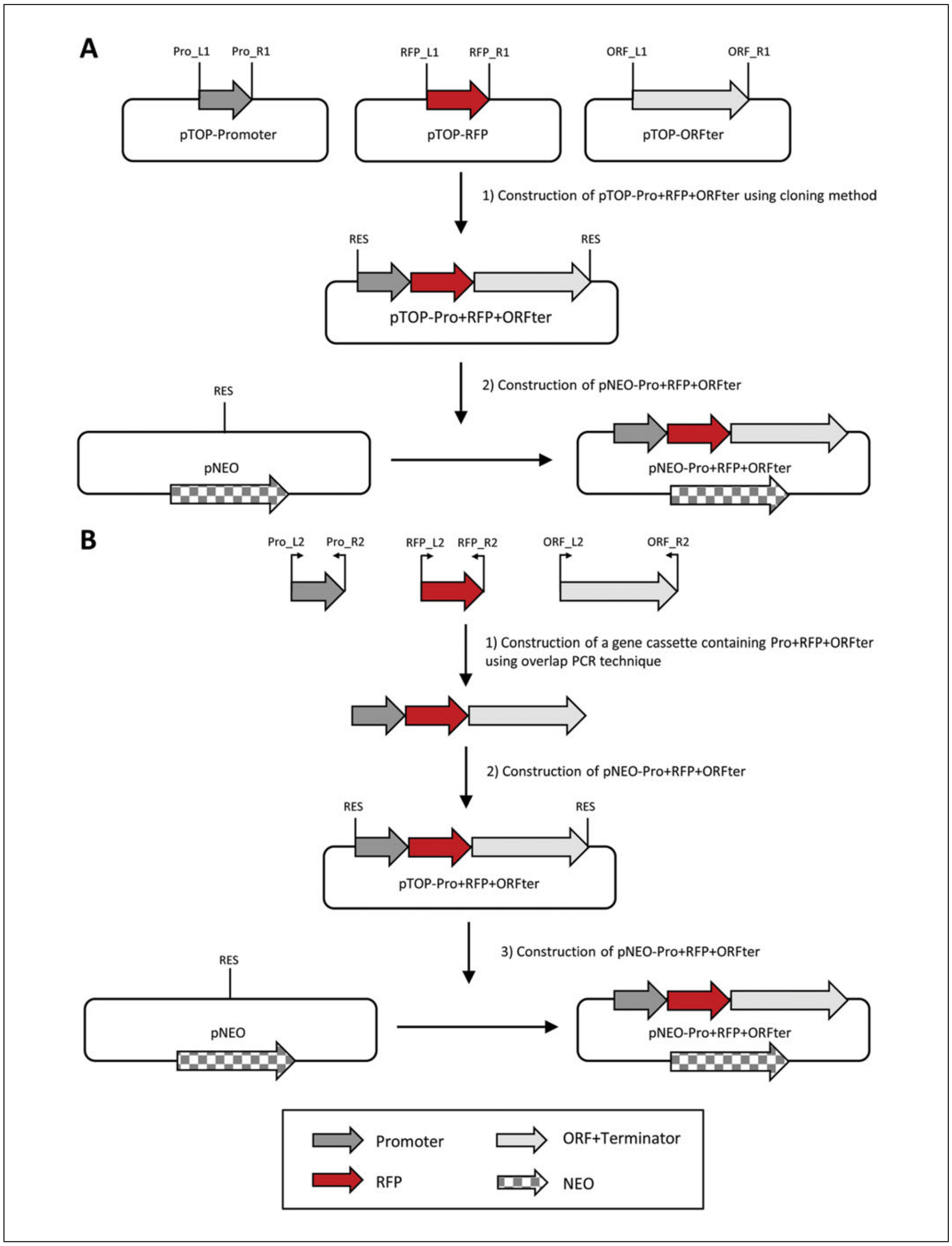

Figure 7 Strategy for construction of strains containing the N-terminal epitope tag by plasmid complementation. (**A**) To construct the pTOP-Pro+RFP+ORFter plasmid, each promoter (Pro) and ORF region without the start codon and terminator (ORFter) of the “target gene”, and the RFP region (RFP) were cloned into the pTOP-V2 plasmid. Next, a designated restriction enzyme-digested PCR insert of the pTOP-Pro+RFP+ORFter plasmid was subcloned into the plasmid pNEO to construct pNEO-Pro+RFP+ORFter. The linearized pNEO-Pro+RFP+ORFter was introduced into the “target gene” mutant. (**B**) Each promoter and ORF region without the start codon and terminator of the “target gene”, and the RFP region was amplified with the indicated primer pairs, respectively. Next, a gene cassette containing Pro+RFP+ORFter was amplified using overlapping PCR. The gene cassette was cloned in the pTOP-V2 plasmid to generate the pTOP-Pro+RFP+ORFter plasmid. After the sequence was confirmed to be error-free, a designated restriction enzyme-digested PCR insert of pTOP-Pro+RFP+ORFter was subcloned into the pNEO plasmid to generate pNEO-Pro+RFP+ORFter. The linearized pNEO-Pro+RFP+ORFter was introduced into the “target gene” mutant. RES indicates the restriction enzyme site for subcloning.

3c. Amplify the RFP region using pNEO-RFPht as the template with a primer pair (Figure 4).

10× ExTaq buffer: 5 µl (final 1× ExTaq buffer)
2.5 mM dNTP: 4 µl
Primer RFP_L1/RFP_R1 (10 pM): 2 µl each
Template: 400 to 500 ng *C. neoformans* genomic DNA
ExTaq: 1.25 U
Distilled water: up to 50 µl.

4c. Set up the PCR as follows:

1 cycle:	2 min	95°C (initial denaturation)
35 cycles:	30 sec	95°C (denaturation)
	30 sec	55°C (annealing)
	XX	72°C (XX depends on the amplicon size, considering polymerase speed to be 1 kb/1 min)
1 cycle:	10 min	72°C (final extension).

5c. Clone each PCR product into the pTOP-V2 plasmid to generate the plasmid pTOP-Promoter, pTOP-RFP, and pTOP-ORFter, respectively (Figure 7A).

6c. Verify sequence error(s) of the PCR product.

7c. After confirming the DNA sequence, construct the plasmid pTOP-Pro+RFP+ORFter by restriction enzyme digestion and ligation using the inserts of the respective plasmids (Figure 7A).

8c. Subclone a designated restriction enzyme-digested cloning insert of the pTOP-Pro+RFP+ORFter into the pNEO plasmid to construct pNEO-Pro+RFP+ORFter (Figure 7A).

9c. Insert the designated restriction enzyme-digested pNEO-Pro+RFP+ORFter into the “target gene” mutant via biolistic transformation.

10c. Perform diagnostic PCR with the designated primer pairs to determine targeted or ectopic integration.

11c. After verification of targeted integration, perform phenotypic analysis to determine whether all the mutant phenotypes are restored to wild type and confirm the functionality of the tagged protein.

REAGENTS AND SOLUTIONS

CTAB buffer

50 ml of 1 M Tris·Cl solution, pH 7.0
20.44 g NaCl
10 ml of 0.5 M EDTA, pH 8.0
5 g CTAB
5 ml 2-mercaptoethanol
Add deionized water up to 500 ml
Store up to 6 months at room temperature

Denaturation buffer

43.83 g NaCl
10 g NaOH
500 ml deionized water
Store up to 7 days at room temperature

Modified Church buffer

1 ml of 0.5 M EDTA, pH 8.0
33.5 g Na_2HPO_4
5 g hydrolyzed casein
35 g SDS
1 ml 85% H_3PO_4
Add deionized water up to 500 ml

Check precipitation before use. If precipitation occurs, dissolve it by heating.

Store up to 6 months at room temperature

Neutralization buffer

43.83 g NaCl
30.37 g Tris
Adjust to pH 8.0 with HCl
Add deionized water up to 500 ml
Store up to 7 days at room temperature

SSC solution, 20×

88.23 g trisodiumcitrate ($Na_3C_6H_5O_7$)
175.32 g NaCl
Adjust to pH 7.0 with HCl
Add deionized water up to 1 liter
Store up to 6 months at room temperature

TENTS buffer

50 ml of 1 M Tris·Cl solution, pH 7.5
1 ml of 0.5 M EDTA, pH 8
2.92 g NaCl
50 ml of 20% Triton X-100
50 ml of 10% SDS
349 ml deionized water (500 ml final volume)
Store up to 6 months at room temperature

Washing buffer I

100 ml of 20× SSC solution (see recipe)
10 ml of 10% SDS
Add deionized water up to 1 liter
Prepare fresh

Washing buffer II

20 ml of 20× SSC solution (see recipe)
10 ml of 10% SDS
Add deionized water up to 1 liter
Prepare fresh

YPD broth (or agar)

20 g peptone
10 g yeast extract
900 ml deionized water
Autoclave
100 ml glucose solution (20%)

After autoclaving, allow the temperature of the solutions to decrease to 55°C, and then pour 100 ml glucose solution (20%) into the broth. For preparing plates, add 20 g agar to the broth mixture before autoclaving. If required, add other supplements

(1 M sorbitol) and antibiotics such as nourseothricin, G418, and hygromycin B. Pour agar into sterile petri dishes.

COMMENTARY

Background Information

Cryptococcus neoformans and *Cryptococcus gattii* are major etiological agents of fatal fungal diseases and cause more than 1,000,000 infections, leading to ~600,000 deaths per year (Park et al., 2009). *C. neoformans* causes life-threatening meningoencephalitis, mainly in immunocompromised individuals, whereas *C. gattii* causes fungal pneumonia in both immunocompetent and immunocompromised populations. Recently, the *C. neoformans* and *C. gattii* species complex was further classified into three (*C. neoformans*, *C. deneoformans*, and *C. neoformans* ×, *C. deneoformans* hybrid) or five (*C. gattii*, *C. deuterogattii*, *C. bacillisporus*, *C. tetragattii*, and *C. decagattii*) species, respectively, based on phylogenetic analysis (Hagen et al., 2017; Kwon-Chung et al., 2017). The *C. neoformans* H99 strain has been mainly used as a reference strain for genetic, molecular, and pathogenic studies because *C. neoformans* accounts for 90% of global *Cryptococcus* infections.

Since the announcement of the *C. neoformans* H99 genome sequence, diverse molecular and genetic tools have been developed for understanding the underlying pathogenicity mechanisms of *C. neoformans* (Heitman, Casadevall, Lodge, & Perfect, 1999; Loftus et al., 2005). Among these, genetic manipulation of target genes is the first step towards understanding the pathobiology of *C. neoformans*. Towards this objective, electroporation was first used to introduce exogenous DNA into *C. neoformans* (Edman & Kwon-Chung, 1990). Although a linear vector shows higher frequency of chromosomal integration than its supercoiled form, the efficiency of electroporation was poor (Edman & Kwon-Chung, 1990). Toffaletti et al. demonstrated that gene delivery via biolistic transformation results in high-frequency integration and produces more stable transformants than electroporation (Toffaletti et al., 1993).

Use of the right selection marker is critical for selecting positive transformants containing the exogenous gene during genetic manipulation. In 1990s, auxotrophic markers such as *URA5* and *ADE2* were used for construction of mutants and its complemented strains (Edman & Kwon-Chung, 1990; Toffaletti et al., 1993). However, strains mutated for auxotrophic markers are inappropriate for studying fungal virulence because the nutritional environment in the infected animal may influence the proliferation or pathogenicity of the auxotrophic wild-type strains. Furthermore, the expression level of the auxotrophic marker may vary with the site of genomic integration. Lower levels of pathogenicity of auxotrophic *Candida albicans* mutants compared to the control prototrophic strains corroborate this hypothesis (Kirsch & Whitney, 1991). Therefore, several antibiotic resistance genes such as *HYG*, *NAT*, and *NEO* have been used as dominant selection markers in *C. neoformans* (Cox, Toffaletti, & Perfect, 1996; Hua, Meyer, & Lodge, 2000; McDade & Cox, 2001).

Instead of using cloned plasmids containing targeting alleles, PCR-amplified targeting alleles have been generally used in *S. cerevisiae* and *C. albicans* as it is more cost-effective and less time-consuming (Baudin, Ozier-Kalogeropoulos, Denouel, Lacroute, & Cullin, 1993; Lorenz et al., 1995; Wilson, Davis, & Mitchell, 1999). In *C. neoformans*, overlapping PCR has been commonly used to construct gene disruption cassettes containing targeting alleles with dominant selection marker insertion (Davidson et al., 2002). The split marker disruption strategy involves fusion of gene disruption cassettes containing truncated but overlapping selection markers (Fairhead, Llorente, Denis, Soler, & Dujon, 1996). This method decreases the integration of the gene disruption cassette via non-homologous recombination and the frequency of multiple or tandem integration, thereby reducing the number of false-positive transformants (Chung & Lee, 2015), and has therefore been widely used in filamentous fungi (Choquer et al., 2005; Lin, Yang, Wang, & Chung, 2010). Although the split *URA5* auxotrophic marker system was developed in *C. neoformans* (Fu, Hettler, & Wickes, 2006), it cannot be utilized in prototrophic wild-type *C. neoformans* strains. Kim et al. developed a split marker strategy with dominant selection marker, which method has been successfully utilized to construct large-scale transcription factor and kinase mutant libraries (Jung et al., 2015; Kim et al., 2009; Lee et al., 2016).

Although gene disruption by homologous recombination and biolistic transformation have been critical for characterizing the function of genes in *C. neoformans*, these methods are limited to non-essential genes. Therefore, a conditional and inducible promoter replacement method has been broadly utilized to confirm gene function in model yeasts. In *C. neoformans*, several inducible promoters have been developed. The *GAL7* promoters from *C. neoformans* H99 and *C. deneoformans* JEC21 strains (also known as serotype D of *C. neoformans*) can be induced by galactose treatment and suppressed by glucose treatment (Ruff, Lodge, & Baker, 2009; Wickes & Edman, 1995). The *CTR4* (copper transporter 4) promoter is tightly repressed by copper treatment and induced by a copper chelating agent (Ory, Griffith, & Doering, 2004). Moreover, the *MFα1* promoter is induced on the V8 media (del Poeta et al., 1999). However, environmental changes such as nutritional variation and specific cation deficiency used for inducing promoter-fused genes may result in unexpected target gene-independent effects. Therefore, use of a constitutively overexpressed promoter, which is not affected by environmental changes, could be an alternative for studying the function of essential genes. Towards this end, *ACT1* (actin), *GPD1* (glycerol-3-phosphate dehydrogenase), and histone H3 promoters have been most popularly used in *C. neoformans* (O'Meara et al., 2010; Panepinto, et al., 2010; Waugh et al., 2002). Histone H3 promoter from the *C. deneoformans* JEC21 genome was used for localization of Hxk2 and Rim101 fusions with fluorescent proteins (Idnurm, Giles, Perfect, & Heitman, 2007; O'Meara et al., 2010). The histone H3 promoter was further utilized for characterization of essential genes and for epigenetic studies (Jung, Kang, & Bahn, 2013; Jung, So, & Bahn, 2016; Lee et al., 2014; Lee et al., 2016; Yang et al., 2017; Yang et al., 2012).

Unlike the advanced genetic tools used for gene disruption and overexpression, protein-tagging systems are not well-developed in *C. neoformans* because of the lack of autonomously replicating stable plasmids. So et al. (2017) recently constructed 12 tagging plasmids containing different epitopes (4× FLAG and 6× HA) and fluorescent proteins (GFP and mCherry) with combination of different dominant selection markers (*NAT, NEO*, and *HYG*). These plasmids can be used for co-immunoprecipitation, immunoblotting, and determination of the localization of target proteins (So et al., 2017; Yang et al., 2017).

Critical Parameters

Considering that repeated serial passages of *C. neoformans* may cause unexpected mutations, all strains used for biolistic transformation should be freshly cultured from a frozen stock (within 1 week). PCR-amplified products (3 to 5 µg) such as gene disruption cassettes, overexpression gene cassettes, and C-terminal epitope tagging cassettes are recommended for biolistic transformation, whereas more than 10 µg of the digested plasmid is required for biolistic transformation.

Troubleshooting

Possible problems arising from the protocols in this unit and their solutions are described in Table 2.

Understanding Results

The *SHO1* disruption cassette was constructed using split marker/DJ-PCR strategy. For the first round of PCR, primer pairs L1 (5′-ATCTCCCAATCTCCCGAAG-3′)/L2 (5′-TCACTGGCCGTCGTTTTACAAGAAAGACTGGGTGTCGC-3′) and R1 (5′- CATGGTCATAGCTGTTTCCTGACACCCGCTGGTATTACAG-3′)/R2 (5′-AAGTTTTCCTCCACTCGCC-3′) were used for amplification of the 5′-and 3′-flanking regions of the gene, respectively (Figure 8A). Primers M13Fe and M13Re were used for amplification of the *NAT* selection marker using pNAT-STM as the template. In the second PCR, primer pairs L1/B1455 and R2/B1454 were used for amplification of the 5′- and 3′-regions of the gene disruption cassettes, respectively. The gene disruption cassette was introduced into the *C. neoformans* H99 strain by biolistic transformation. The correct genotype of the positive transformants initially screened by diagnostic PCR was verified by Southern blot analysis using genomic DNAs of the positive transformants digested with *Eco*RI and *Sac*I restriction enzymes (Figure 8B) (Kim et al., 2015).

To construct the constitutive *CGP1* overexpression strain, primers OE-L1 (B3714, 5′- GAGCAGCAAAGATTTCGC-3′) and OE-L2 (B6652, 5′-CACTCGAATCCTGCATGCTTAATGAGATTACAAGGTA-3′) for the 5′-flanking region of *CPG1,* and OE-R1 (B6653, 5′- ACCACAACACATCTATCACATGTCACGCTTACCTCAAC-3′) and OE-R2 (B6654, 5′- GAGATGAGGCAACAGAAGC-3′) for the 5′-exon region of *CGP1* were used in the first round of PCR. The *NAT* marker with the H3 promoter region was amplified with primer pairs B4017/B4018 using

Table 2 Troubleshooting Guide for Genetic Manipulation of *C. neoformans*

Problem	Possible cause	Possible solution
PCR amplification		
Nonspecific amplification in PCR	Primer design problem	Try to make the melting temperature (T_m) of the primers between 55°C and 65°C and GC contents of the primers between 45% and 60%.
Biolistic transformation		
Low frequency of transformation	Abnormal explosion of rupture disk	Transformation is performed with *C. neoformans* at 1350 psi under a vacuum of 28.5 in Helium gas. Make sure the rupture disk holder is tightly locked to be bombarded at 1350 psi.
	High concentration of antibiotics in the selection media.	The recommended concentration of antibiotics is followed. (nourseothricin: 100 µg/ml, G418: 50 µg/ml, or hygromycin B: 150 µg/ml). Depending on the number of transformants appeared on the selection medium, you could change concentration of antibiotic.
No positive transformants	Essentiality of target gene	If transformants do not appear with repeated trials, check the essentiality of its ortholog. If information is not available, you try to construct the conditional mutant by using *CTR4* promoter replacement to check essentiality of gene.
	Primer design	Make sure that gene disruption cassette does not affect neighboring genes.
Modified "Smash & grab" mini-preparation		
Small amount of gDNA	Degree of powdering	Make sure that beads beating is sufficient to disrupt cells.
Southern blot		
Weak blotting signal	Insufficient probe concentration or size	Increase in the amount of probe PCR product. The probe size could be more than 1 kb.
	Inadequate DNA transfer to membrane	Make sure that the paper towel piled up for capillary transfer has collapsed or that the absorbed buffer has flowed backwards.

(Continued)

Table 2 *Continued*

Problem	Possible cause	Possible solution
	Lower concentration of digested DNA	Make sure isolated DNA concentration is sufficient. More than 50 μg of DNA is needed to be probed.
	Lower activity of radioisotope	Use radioisotope within two times of half-life.
Aberrant bands	Incomplete DNA digestion	Put more restriction enzyme based on the unit and give enough time to be digested.
	Genomic DNA contamination	Extract genomic DNA from new samples.
Splotch and speck	Inadequate pre-hybridization time	Increase pre-hybridization time.
	Probe not evenly mixed	Put the labelled probe into the hybridization buffer, not direct to the membrane.

pNAT-H3 as the template. In the second round of PCR, the 5′ and 3′ regions of the constitutive *CGP1* overexpression cassettes were amplified with primer pairs OE-L1/B1455 and OE-R2/B1454, respectively, using the DJ-PCR strategy (Figure 8C). The constitutive *CGP1* overexpression cassette was introduced into the *C. neoformans* H99 strain. The expression levels of *CGP1* were monitored by northern blot analysis using total RNAs of independent *CGP1* overexpression strains with *CGP1* specific probe (Figure 8D).

The *ACA1:4× FLAG* tagging strains were constructed using pNEO-4×FLAGht. In the first round of PCR, primer pairs FLAG-L1 (B6559, 5′-CAAGTCCTTCATTGCGTTG-3′)/FLAG-L2 (B6570, 5′-GTAATCATCTGG CGCGCCGCATGCTCCTGAGTGCTCTAC AAT-3′) and FLAG-R1 (B6561, 5′-CCACT CGAATCCTGCATGCATAGAAAGAAGAA AGAGAGTTC-3′)/FLAG-R2 (B6562, 5′-GA TAGATACCGAGAATGGGG-3′) were used for amplification of the C-terminal exon region of *ACA1* and *ACA1* terminator regions, respectively. Primers B5536 (5′-GCGGCCGC AGCATGCGGCGCGCCAGAT-3′) and B354 (5′-GCATGCAGGATTCGAGTG-3′) were used to amplify the *4× FLAG-NEO* construct. In the second round of PCR, the 5′ and 3′ regions of the *ACA1:4× FLAG-NEO* construct were amplified with primer pairs FLAG-L1/B1886 and FLAG-R2/B1887, respectively, using the first round PCR products as templates. The *ACA1:4× FLAG-NEO* constructs were introduced into the *C. neoformans* H99 strain (Figure 8E). The correct genotype of the *ACA1:4× FLAG* tagging strain was verified by Southern blot analysis. The production of Aca1:4× FLAG-tagged protein was confirmed by western blot analysis using monoclonal anti-FLAG M2 antibody (Sigma-Aldrich, cat. no. F3165). The anti-β-actin antibody (Santa Cruz Biotechnology Inc., cat. no. sc-69879) was used for detecting β-actin, the protein loading control (Figure 8F) (So et al., 2017).

Time Considerations

Isolation of *C. neoformans* takes 2 to 3 days after streaking onto agar-based YPD media at 30°C. Slightly longer time might be required for isolation from plates containing antibiotics such as nourseothricin, neomycin, and hygromycin B. The first and second round PCRs for the construction of target gene deletion cassette, constitutive overexpression promoter cassette, and C-terminal epitope tagging cassette take 3 to 4 hr each. Biolistic transformation is completed in 8 hr. The *C. neoformans* colonies are visible on the selection plate at 3 to 4 days post-biolistic transformation. Longer incubation time is required if transformants have a growth defect. The modified "smash & grab" mini-preparation and diagnostic PCR can be completed within 1 day. Five or seven days are required for verification of the correct genotype of transformants, starting from genomic DNA isolation of transformants to development of the autoradiography film.

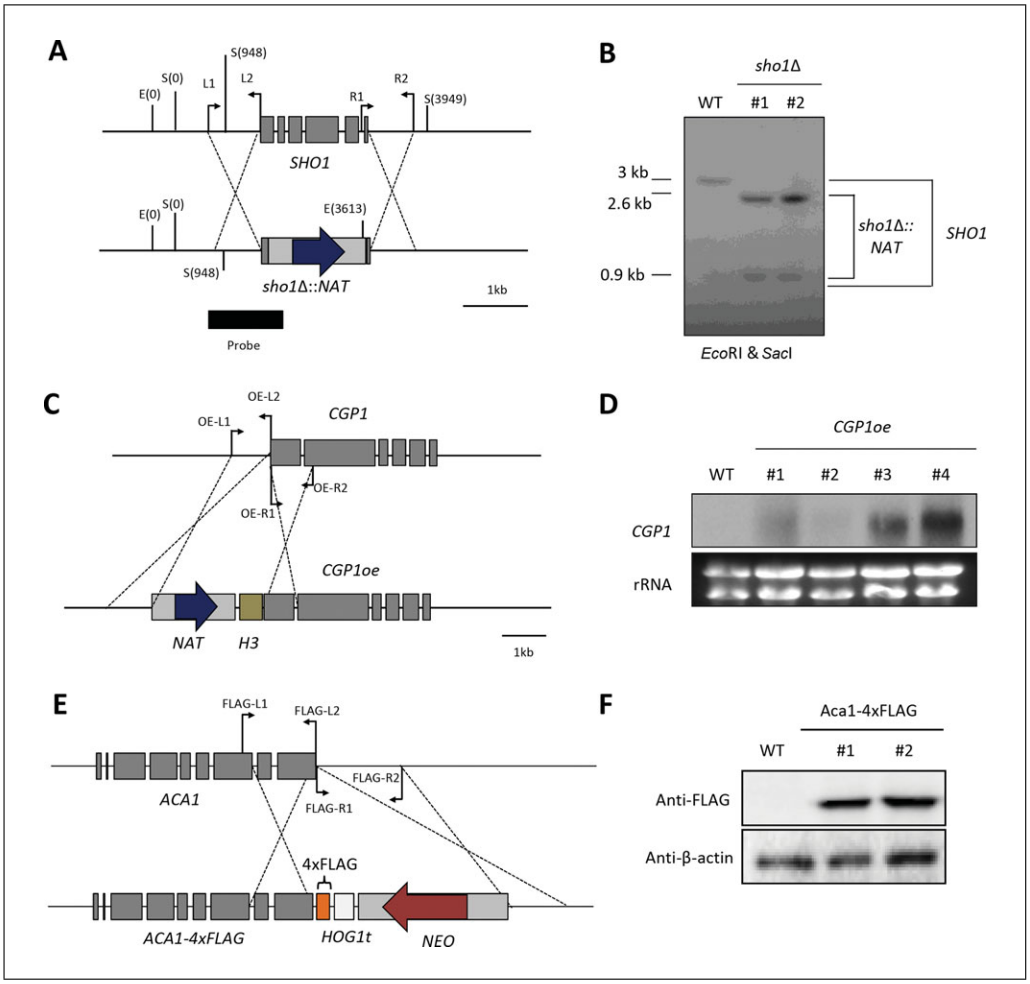

Figure 8 Examples of genetic manipulation of *C. neoformans* (**A**, **C**, and **E**). Diagrams showing target disruption of *SHO1*, and construction of the constitutive *CGP1* overexpression and *4*× *FLAG*-tagged *ACA1* strain. (**B**) Correct *SHO1* disruption was confirmed by Southern blot analysis using genomic DNAs digested with the EcoRI and SacI. (**D**) Northern blot analysis for *CGP1* expression in WT and four independent *CGP1oe* (*CGP1* overexpression) strains (#1 ~ #4). Ethidium bromide stained-rRNA was used as loading controls. (**F**) The two strains harboring Aca1:4× FLAG were verified by immunoblot analysis using an anti-FLAG antibody. The WT was used as a negative control for FLAG-tagging. The anti-β-actin was used as a protein loading control.

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Internet Resources

https://fungidb.org/fungidb/

For genetic manipulation of the gene of interest in C. neoformans, information regarding genomic structures and sequences can be obtained from the Fungal and Oomycete Genomics Resource