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RNA Interference in *Cryptococcus neoformans*

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Abstract

RNA interference (RNAi) is an experimental technique used to suppress individual gene expression in eukaryotic cells in a sequence-dependent manner. The process relies on double-stranded RNA (dsRNA) to target complementary messenger RNA for degradation. Here, we describe two plasmid-based strategies we have developed for RNAi in *Cryptococcus neoformans*. The pFrame vector utilizes the *ACT1* promoter to enable the constitutive synthesis of hairpin RNA (hpRNA), the stem of which constitutes the dsRNA trigger. The pIBB103 vector relies on convergent, inducible *GAL7* promoters to independently drive the synthesis of the sense and antisense strands of the interfering sequence; these strands anneal to form the initiating dsRNA molecule. Both vectors are designed to co-silence a “sentinel” gene with an easily scored phenotype to help identify clones in which RNAi is most effective. We provide guidelines for selecting a suitable interfering sequence to trigger RNAi in *C. neoformans* and describe the steps for subcloning into either vector, transforming *C. neoformans* by electroporation, screening clones for RNAi-related phenotypes, and evaluating the efficacy and specificity of gene silencing by RNAi.

Keywords

Cryptococcus neoformans; RNA interference; Gene silencing; Gene expression

1. Introduction

RNA interference (RNAi) is a way to experimentally suppress the expression of specific genes in eukaryotic cells by using double-stranded RNA (dsRNA) to target homologous messenger RNA (mRNA) for degradation. The technique harnesses components of the post-transcriptional gene silencing machinery that is found in most eukaryotes, including pathogenic fungi (1), and that has been implicated in gene regulation (2) and protection against viruses and mobile genetic elements (3). Briefly, RNAi is triggered by dsRNA that has been either directly introduced into a cell by transformation or synthesized intracellularly from a plasmid or a chromosomally integrated construct. The endogenous ribonuclease Dicer cleaves the dsRNA molecules into 20–30-bp pieces (4). The resulting small interfering RNA (siRNA) fragments are incorporated into an RNA-induced silencing complex (RISC) that catalyzes the sequence-specific cleavage of mRNA. Since its discovery nearly two decades ago, RNAi has been implemented in a variety of fungi (5, 6), including *Cryptococcus neoformans*.

Early evidence that RNA-mediated silencing functions in *C. neoformans* was provided in 2002 by Gorch et al. (7). These investigators successfully suppressed the synthesis of calcineurin A (*CNA1*) in serotype D and laccase (*LAC1*) in serotype A using antisense repression, a technique similar to RNAi but triggered by single-stranded RNA complementary to endogenous mRNA. The same year, we reported successful silencing of

CAP59 and *ADE2* by RNAi in serotype D (8). For these studies, we used an episomally expressed hairpin RNA (hpRNA) consisting of the sense and antisense strands of the target sequence connected by a linker (Fig. 1). The complementary nature of the antiparallel strands allows them to anneal intramolecularly to form a double-stranded duplex RNA which is subject to cleavage by Dicer. Since then, we have implemented a simpler system utilizing convergent promoters to drive transcription of both the sense and antisense strands of the target sequence (Fig. 2). The individual transcripts anneal intermolecularly to form an RNA duplex that triggers RNAi. We and others have employed both the hairpin (8–12) and convergent promoter (13) RNAi constructs to study genes implicated in *C. neoformans* virulence. Our discussion regarding RNAi in this organism will, therefore, focus on these two plasmid-based methods.

Over the past several years, the sequenced and annotated genomes of *C. neoformans* strains representative of serotypes A, B, and D have been made available, facilitating genetic manipulation in this pathogen (see Note 1). Yet despite concomitant improvements in the efficiency of gene disruption in this organism (14–16), RNAi offers several advantages that are not possible or difficult to achieve with gene disruption alone. For example, intracellular synthesis of interfering dsRNA can be driven by various promoters, potentially enabling either constitutive (17, 18) or inducible (19–21) down-regulation that restricts RNAi to a specific time frame or growth phase. Such transient silencing can be used as an alternative to conditional mutations in studying the function of essential genes (5). Furthermore, multiple genes can be silenced in parallel using combinations of dsRNA directed against each transcript. Since RNAi operates at the post-transcriptional level, members of the same gene family or homologous genes in different strains can be targeted using a single motif (22); likewise, specific splice variants can be selectively inhibited by targeting a unique sequence not shared by the other transcripts (23). An exciting application of RNAi that has begun to gain momentum is the capacity for high-throughput library screening in genome analysis (24–26) and the identification of genes involved in host–pathogen interactions (27).

In contrast to gene deletion, RNAi does not completely eliminate the expression of the target gene. Consequently, the extent to which a gene is silenced depends on multiple factors, including the kinetics of target gene expression, the efficiency of the interference itself, the stability of the gene product, and the threshold for gene function in the process of interest (5, 28). Although in many cases RNAi does produce a null phenotype, it may also generate a range of partial phenotypes if the targeted gene is insufficiently silenced. To assess the efficacy of RNAi, it is necessary to compare the extent of interference in multiple clones. A qualitative comparison can be made on the basis of observable phenotypes, while a more quantitative approach involves analyzing the abundance of the targeted mRNA by quantitative PCR (qPCR) or RNA blotting, and/or evaluating the abundance of the corresponding gene product by immunodetection or a functional assay.

The partial phenotypes produced by incomplete silencing may help to elucidate the roles of essential genes or correlate expression level with observed phenotype. However, incomplete silencing may also result in the lack of a detectable phenotype. A reporter strategy has been used by us in *C. neoformans* (8) and by others in *Histoplasma capsulatum* (29) and *Blastomyces dermatitidis* (30) to compare the extent of interference in specific transformants. This approach involves co-silencing the intended target gene in parallel with an unrelated “sentinel” gene. This sentinel should have an easily scored phenotype and

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¹The genomes of *C. neoformans* var. *neoformans* JEC21 and B-3501A (capsule serotype D) are available through the Genome Project database at the National Center for Biotechnology Information (NCBI) at <http://www.ncbi.nlm.nih.gov/genomes/leuks.cgi>. Those of *C. neoformans* var. *grubii* H99 (capsule serotype A) and *C. neoformans* var. *gatii* R265 (capsule serotype B) are available through the Fungal Genome Initiative at the Broad Institute of MIT and Harvard at <http://www.broadinstitute.org/science/projects/projects>.

preferably be known to be efficiently silenced. Although this method generally helps to identify clones experiencing stronger interference, silencing of the sentinel and target genes still occurs independently and may not correlate in all cases.

In any RNAi experiment, it is critical to confirm that an observed phenotype results exclusively from silencing of the intended target gene. Since the mechanism of RNAi is sequence-dependent instead of locus-dependent, RNAi may trigger the suppression of genes whose mRNA contains a sequence similar to the interfering dsRNA (5, 28). Such “off-target” silencing can potentially be minimized by selecting the interfering sequence from within a unique region in the intended target gene, and ensuring that this sequence has low homology to other endogenous genes. One way to confirm the specificity of RNAi is to independently target different regions of the same mRNA to see whether the observed phenotype is recapitulated. In addition, it may be possible to complement an RNAi phenotype with a mutant version of the target gene that has been engineered to differ in sequence from the interfering dsRNA (a technique termed “gene resurrection”) (5, 28). To verify whether the plasmid or experimental conditions themselves potentially cause unintended silencing, a mock transformation should be performed using the vector without the interfering sequence.

In this chapter, we detail the use of the two plasmid-based strategies we have developed for RNAi in *C. neoformans*. The pFrame vector (Fig. 3, Genbank accession number HM352736) is based on the hairpin design we initially used to implement RNAi in this organism (8). It permits constitutive synthesis of an hpRNA construct flanked by the *ACT1* promoter (18) and *GAL7* terminator (19), and further offers the option of co-silencing endogenous *ADE2* as a sentinel for RNAi. The hpRNA construct consists of 510 bp of the *ADE2* coding sequence, followed by a custom interfering sequence, a 250-bp spacer fragment derived from GFP (to serve as the loop of the hairpin), the reverse complement of the custom fragment, and the reverse complement of the *ADE2* fragment (the spacer is an exogenous sequence with no homology to endogenous genes to avoid potential silencing from the loop itself). To simplify cloning, two segments of the *E. coli* β -glucuronidase (*gusA*) gene have been inserted as placeholders for the two antiparallel segments of the custom interfering sequence. Cloning into pFrame is achieved by sequentially excising each *gusA* fragment and replacing it with the selected interfering sequence in the appropriate orientation. Following transformation of *C. neoformans* with the fully assembled vector, clones can be screened for efficacy of RNAi based on colony pigmentation on low-adenine medium: Increased suppression of *ADE2* causes greater accumulation of purine precursors and results in a brighter red colony color (8, 31). It should be noted that the *URA5* auxotrophic marker (32) included on the plasmid for selection in *C. neoformans* limits the use of this vector to *ura5* mutant strains (see Note 2). The two-step cloning required to assemble the hairpin constructs can be time-consuming, but hpRNA may induce more consistent RNAi than the convergent promoter strategy described below (5).

The vector pIBB103 (Fig. 4, Genbank accession number HQ455038) overcomes several technical shortcomings of pFrame (33). It utilizes two convergent, galactose-inducible *GAL7* promoters (each paired with a *GAL7* terminator) to independently drive strong transcription of the sense and antisense strands of the interfering construct with little basal activity (19). One-step cloning of the target sequence into an *SpeI* restriction site saves time and permits the construction of RNAi libraries. The inclusion of a Geneticin® (G418) resistance marker (34) makes the vector compatible with most *C. neoformans* strains (see Note 2), and cryptococcal telomeric repeats improve transformation efficiency and

²In our experience, RNAi is considerably less effective in serotype A than in serotype D, which we suspect results from the significantly lower expression of the interfering dsRNA from the serotype A *GAL7* and *ACT1* promoters (21).

intracellular maintenance of the plasmid (35). These repeats are separated by an ~800 bp fragment derived from the bacterial transposon Tn903, which may be released by digestion with the endonuclease I-*SceI* to expose the telomeric repeats at both ends of the linearized plasmid. A 251-bp fragment derived from serotype D genomic DNA mediates co-silencing of endogenous *URA5* and offers the advantage of making RNAi a selectable trait: Transformants can be screened either by negative selection on media lacking uracil or positive selection on media containing 5-fluoroorotic acid (5-FOA) (32, 33). Notably, even weak suppression of *URA 5* allows growth on 5-FOA (albeit at a growth rate slightly slower than that of a *ura5* mutant), without significant inhibition of growth on uracil-deficient media. In the event that targeting *URA5* as a sentinel proves inefficient, we suggest alternatively targeting *ADE2*. The broader range of phenotypes produced from partial suppression of *ADE2* should allow a better assessment of the relative efficacy of RNAi.

A critical first step for implementing RNAi in *C. neoformans* is the choice of a suitable interfering sequence that is specific for the gene being targeted. Once selected, the interfering sequence is amplified by PCR using primers that introduce the appropriate restriction enzyme sites for subcloning into the vector of choice. Next, the fully assembled vector is linearized by restriction enzyme digestion to improve the efficiency of transformation (36) and electroporated into *C. neoformans* (37). After electroporation of the linearized DNA, transformants are selected by growth on appropriate media and screened for suppression of the RNAi sentinel by replica-plating onto suitable media. Those transformants that exhibit inhibition of the sentinel are further assessed for suppression of the target gene(s) by phenotypic analysis. For transformants of interest, the extent and specificity of interference are evaluated as mentioned above.

2. Materials

Prepare all reagents using distilled water, unless noted otherwise. Distilled and deionized water (e.g., from a Milli-Q system) will be referred to as ultrapure. Room temperature (22–25°C) will be abbreviated as RT. All media and agar plates should be prepared using standard sterile technique for microbiological work. Unless noted otherwise, agar plates can be made from any of the media recipes (see Note 3).

2.1. Preparation of the pFrame Vector for Triggering RNA Interference

1. Access to the internet to obtain the coding sequence of the target gene and perform bioinformatic operations (see Note 1).
2. A suitable DNA sequence editor (e.g., the free Serial Cloner application from Serial Basics or the DNASTar Lasergene® sequence analysis software suite).
3. Standard PCR reagents.
4. PCR purification kit (e.g., QIAquick® from Qiagen).
5. *NdeI*, *AvrII*, *XhoI*, *BglII*, and *NotI* restriction enzymes and appropriate buffers.
6. T4 DNA ligase with appropriate buffer.
7. Gel extraction and spin miniprep kits (e.g., QIAquick® and QIAprep® from Qiagen).
8. *Escherichia coli* competent cells.

³Add 20 g of Bacto agar per L of medium, along with a stir bar and the other media components to an Erlenmeyer flask with a capacity at least twofold greater than the intended volume. Autoclave for 20–30 min, cool for 20–25 min by gently stirring at RT, then pour 25 mL per petri plate. Leave to solidify overnight at RT, then seal in plastic wrap and store at 4°C to prevent microbial growth.

9. 1,000× ampicillin solution (100 mg/mL), prepared in water and filter-sterilized. Store in 500 µL aliquots at −20°C.
10. LB (Luria-Bertani) medium: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl in water. Stir until mixed completely, then aliquot desired volume to bottles or flasks and autoclave for 25 min. Store at RT.
11. LB agar plates with 100 µg/mL ampicillin: Prepare 1 L (see Note 3) using the above recipe for LB medium. Add 1 mL of 1,000× ampicillin solution to the cooled agar after autoclaving and stir for 5 more minutes before pouring into petri dishes.
12. 100% and 70% (v/v) ethanol prepared in ultrapure water, both chilled at −20°C.
13. 3 M sodium acetate: Dissolve 40.8 g of sodium acetate in 80 mL of ultrapure water. Adjust the pH to 5.2 with glacial acetic acid, then adjust the volume to 100 mL and filter-sterilize. Store at RT.
14. Ultrapure water that has been filter-sterilized or autoclaved.

2.2. Transformation of *C. neoformans* by the pFrame Vector Using Electroporation

1. Sterile 1.5-mL microfuge tubes, 13-mL culture tubes, and 50-mL conical tubes.
2. Ultrapure water that has been filter-sterilized or autoclaved, and chilled to 4°C.
3. Electroporation buffer (EB): 10 mM Tris–HCl pH 7.5, 1 mM MgCl₂, 270 mM sucrose. Prepare in ultrapure water, filter-sterilize, and chill at 4°C.
4. 1 M Dithiothreitol (DTT), prepared in ultrapure water and filter-sterilized. Store in 500 µL aliquots at −20°C.
5. 0.2-cm Electroporation cuvettes (Bio-Rad).
6. YPD (yeast extract–peptone–dextrose) medium: 1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) dextrose in water. Stir until mixed completely, then aliquot desired volume to bottles or flasks and autoclave for 25 min. Store at RT.
7. Complete uracil dropout mix: Use a commercially available mixture or prepare from the individual components (see Note 4).
8. Uracil-deficient medium: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose, and 0.08% (w/v) complete uracil dropout mix in water. Stir until mixed completely and autoclave for 25 min. Store at RT.
9. Uracil-deficient agar plates: Prepare 1 L as described (see Note 3) using the above recipe for uracil-deficient medium.

2.3. Phenotypic Screening of Transformants Using the ADE2 Sentinel of RNAi

1. Flat-tipped toothpicks, autoclaved.
2. 48-pin Replicator and 100% ethanol.
3. Low-adenine uracil-deficient medium: 0.67% (w/v) yeast nitrogen base without amino acids, 2% (w/v) dextrose, 0.08% (w/v) complete adenine and uracil dropout mix, and 10 mg/L of adenine in water. Stir until mixed completely and autoclave for 25 min. Store at RT.

⁴Prepare as described in Unit 13.1 of the Current Protocols in Molecular Biology series (41), omitting the desired nucleotide.

4. Low-adenine uracil-deficient agar plates: Prepare 1 L as described (see Note 3) using the recipe for low-adenine uracil-deficient medium above.
5. Complete adenine and uracil dropout mix: Use a commercially available mixture or prepare from the individual components (see Note 4).

2.4. Extraction of Total RNA and Analysis of mRNA Abundance

1. Uracil-deficient medium: See items 7–9 in Subheading 2.2.
2. 250-mL Erlenmeyer flasks, autoclaved.
3. 1.5-mL Microfuge tubes and 2-mL screw-cap tubes (RNase-free).
4. 0.5-mm Glass beads and Mini beadbeater.
5. TRIzol® reagent. Store at –20°C.
6. Chloroform, isopropanol, and ultrapure water (all RNase-free).
7. 70% (v/v) Ethanol, prepared in RNase-free ultrapure water and chilled at –20°C.

2.5. Preparation of the pIBB103 Vector for Triggering RNA Interference

1. Items 1–4 and 6–14 in Subheading 2.1.
2. *SpeI* and *I-SceI* restriction enzymes and appropriate buffers.

2.6. Transformation of *C. neoformans* by the pIBB103 Vector Using Electroporation

1. Items 1–6 in Subheading 2.2.
2. 20% (w/v) Galactose solution, prepared in water and filter-sterilized. Store at RT.
3. 1,000× G418 solution (100 mg/mL), prepared in water and filter-sterilized. Store in 500 µL aliquots at –20°C.
4. YPG (yeast extract–peptone–galactose) medium with 100 µg/ml G418: For 1 L of medium, combine 10 g of yeast extract and 20 g of peptone in 900 mL of water. Add a stir bar and autoclave for 20–30 min. Cool by gently stirring for 10–15 min at RT, then add 100 mL of 20% galactose solution while stirring. Store at RT. Right before use, add the appropriate volume of 1,000× G418 solution to the desired volume of medium and swirl to mix well.
5. YPG agar plates with G418: Prepare 1 L as described (see Note 3) using the above recipe for YPG medium. Add 1 mL of 1,000×G418 solution to the cooled agar after autoclaving and stir for 5 more minutes before pouring into petri dishes.

2.7. Phenotypic Screening of Transformants Using the *URA5* Sentinel of RNAi

1. Items 1–2 in Subheading 2.3.
2. Uracil-deficient agar plates with galactose and G418: For 1 L of medium, combine 6.7 g of yeast nitrogen base without amino acids, 800 mg of complete uracil dropout mix (see item 7 in Subheading 2.2), and 25 g of Bacto agar in 900 mL of water. Autoclave with a stir bar for 20–30 min, then cool by gently stirring for 20–25 min at RT. Add 100 mL of 20% galactose solution (see item 2 in Subheading 2.6) while stirring, then add 1 mL of 1,000× G418 solution (see item 3 in Subheading 2.6). Stir for 5 more minutes until the solutions mix completely, then pour 25 mL per petri plate and store at 4°C sealed in plastic wrap.

3. 5-FOA solution (10 mg/ml): Dissolve 1 g of 5-FOA in 100 mL of water with gentle stirring and heating, then cool and filter-sterilize. Prepare right before use.
4. 5-FOA agar plates with galactose and G418: For 1 L of medium, combine 6.7 g of yeast nitrogen base without amino acids, 800 mg of complete uracil dropout mix (see item 7 in Subheading 2.2), 12 mg of uracil, and 25 g of Bacto agar in 800 mL of water. Autoclave with a stir bar for 20–30 min, then cool by gently stirring for 15–20 min at RT. Slowly add 100 mL of 20% galactose solution (see item 2 in Subheading 2.6), 100 mL of 5-FOA solution, and 1 mL of 1,000× G418 solution (see item 3 in Subheading 2.6) to the cooled agar. Stir for 5 more minutes until the solutions mix completely, then pour 25 mL per petri plate and store at 4°C sealed in plastic wrap. Protect from light.

3 Methods

3.1. Preparation of the pFrame Vector for Triggering RNA Interference

1. To select a sequence for triggering RNAi, obtain the coding sequence of the target gene from the appropriate database by one of the following methods:
  - a. If the accession number of the target gene is known for a strain whose genome has been sequenced, use it to directly query the appropriate database and retrieve the corresponding locus record (see Note 1);
  - b. If a genomic, cDNA, or protein sequence corresponding to the target gene from another strain or organism is known, use the BLAST sequence alignment tool available through the NCBI (see Note 5) or the Broad Institute to identify the closest homolog in one of the sequenced *C. neoformans* strains.
2. With the help of a sequence editor application, choose a region within the coding sequence of the target gene that satisfies the following criteria (see Note 6):
  - a. The region should preferably be located within the middle of the coding sequence (see Note 7);
  - b. The region should have minimal homology to the coding sequences of other genes in the same strain (see Note 8);
  - c. The region should not exceed 500 bp in length (see Note 9);
  - d. The proportion of A-T base pairs to G-C base pairs should be approximately equal (see Note 10);

⁵The BLAST utility offered by the NCBI can be accessed at <http://blast.ncbi.nlm.nih.gov/>. For more information or instructions on using BLAST, refer to online documentation by clicking the Help tab.

⁶Most of the research on optimizing target fragment selection for RNAi is conducted with respect to siRNA. Although not all of the guidelines established for optimal siRNA selection are directly applicable to longer dsRNA trigger fragments, a detailed discussion and comparison of the current rules and methods can be found in Takasaki (42). Information on using bioinformatic algorithms for optimizing longer dsRNA triggers can be found in Horn and Boutros (43).

⁷The termini of mRNA molecules are often highly structured and bound by regulatory proteins, which may impede the RISC from binding to the mRNA at those locations (44). It is generally recommended to avoid target sequences within 50–100 bp of the start or stop codon.

⁸Depending on the sequence, regions of homology longer than 15–25 bp (the range for a typical siRNA) might lead to off-target silencing (45). We recommend using the BLAST utility for this purpose (see Note 5).

⁹We have found regions of 200–300 bp to be most effective (Hong Liu, unpublished data). A longer sequence may increase the frequency of off-target and partial silencing because of the greater number of different siRNA fragments that can be generated from it by Dicer, each of which might trigger RNAi to a varying extent in different clones.

¹⁰A GC-rich region within the mRNA might adopt a stable secondary structure that could block access of the RISC to the targeted sequence (46).

- e. Avoid repeats longer than 3–5 bp of the same residue (see Note 11);
  - f. The region should not contain the recognition sites for any of the following restriction enzymes: *NdeI*, *BglII*, *AvrII*, *XhoI*, and *NotI* (see Note 12).
3. Design a sense oligonucleotide primer (primer A) that overlaps 20–25 bp of the 5' end of the selected target sequence. Add an *NdeI* restriction site (CATATG) to the 5' end of this sequence, and then add six randomly arranged nucleotides to the 5' end of the resulting primer (see Note 13). Design a second primer (primer B) that differs only by the inclusion of a *BglII* restriction site (AGATCT) instead of the *NdeI* site.
  4. Design an antisense oligonucleotide primer (primer C) that overlaps 20–25 bp of the 3' end of the selected target sequence. Add an *AvrII* restriction site (CCTAGG) to the 5' end of this sequence, and then add six randomly arranged nucleotides to the 5' end of the resulting primer (see Note 13). Design a second primer (primer D) that differs only by the inclusion of an *XhoI* restriction site (CTCGAG) instead of the *AvrII* site.
  5. Amplify the selected target sequence by PCR using primers A and C in one reaction and primers B and D in a second reaction (see Note 14). Use cDNA (see Subheading 3.4) as a template if the target sequence overlaps exon junctions. Otherwise, genomic DNA may be used (38).
  6. Confirm amplification of the desired fragments by resolving 1–5 µL of each reaction (i.e., 20–50 ng of product) by agarose gel electrophoresis (see Note 15).
  7. Purify each fragment using a PCR purification kit and determine the DNA concentration (see Note 16).
  8. Digest 1 µg of the fragment generated using primers A and C with 5–10 U each of *NdeI* and *AvrII*; digest 1 µg of the fragment generated using primers B and D with 5–10 U each of *XhoI* and *BglII*. Incubate for 12–16 h at 37°C (see Note 17) and purify each fragment using a PCR purification kit. Store at –20°C.
  9. Prepare the pFrame vector for cloning: Excise one *gusA* fragment by digesting 1–2 µg of the plasmid with 5–10 U each of *NdeI* and *AvrII* for 12–16 h at 37°C. Gel purify the 8-kb vector backbone using a gel extraction kit.
  10. Mix 30 ng (6 fmol) of the *NdeI*-, *AvrII*-cut plasmid with 5–15 ng (~30 fmol) of the *NdeI*-, *AvrII*-cut target fragment on ice. Add water and ligation buffer up to 20 µL. Add 1 µL of T4 DNA ligase and incubate at RT for 30–60 min.

¹¹Such repeats decrease the specificity of RNAi and may lead to off-target silencing (45).

¹²These sites should be avoided because they are used for cloning into the vector (see Fig. 3 for pFrame or Fig. 4 for pIBB103). If a region lacking a particular restriction enzyme recognition site cannot be found, consider either substituting an enzyme that cleaves to leave a compatible cohesive end or use blunt-end ligation to clone the fragment into the RNAi vector of choice.

¹³The additional nucleotides improve restriction enzyme cleavage at the ends of the PCR product obtained with these primers (refer to the technical reference section in the product catalog from New England Biolabs for more information).

¹⁴A high-fidelity DNA polymerase such as AccuPrime™ *pf*x DNA Polymerase (Invitrogen) should be used to minimize the incorporation of mutations. If the PCR requires a cDNA template, we recommend using a derivative of *Taq* DNA polymerase, such as AccuPrime™ *Taq* DNA Polymerase High Fidelity (Invitrogen).

¹⁵Refer to Unit 2.5A in the Current Protocols in Molecular Biology series for more information (47).

¹⁶A Nanodrop™ (Thermo Scientific) or similar microvolume spectrophotometric device provides a rapid way to quantitate nucleic acids. Alternatively, dilute the DNA sample 10- to 100-fold in water or a suitable buffer, transfer to a spectrophotometer cuvette, and measure the absorbance at 260 nm. By convention, an absorbance value of 1 = 50 ng/µl of dsDNA.

¹⁷Alternatively, the PCR products may first be TA-cloned into a suitable vector such as pCR®2.1-TOPO® (Invitrogen) and subsequently excised with the appropriate restriction enzymes.

11. Transform competent *E. coli* with the ligation reaction according to the supplier's instructions. Plate 20% and 80% of the transformation on LB agar containing 100 µg/mL ampicillin and incubate overnight at 37°C.
12. Screen the transformants by colony PCR or restriction analysis to confirm insertion of the first target fragment, and extract plasmid DNA from a clone containing the desired insert using a spin miniprep kit as specified by the manufacturer.
13. Excise the second *gusA* fragment: Digest 1–2 µg of the plasmid isolated in step 12 with 5–10 U each of *XhoI* and *BglII* for 12–16 h at 37°C. Gel purify the plasmid backbone, ligate to the *XhoI*-, *BglII*-cut target fragment, and isolate the completed plasmid as described in steps 10–12 above. Store at –20°C.
14. Prepare the fully assembled plasmid for electroporation: Digest 1–5 µg (see Note 18) of the vector with 5–10 U of *NodI* for 12–16 h at 37°C, and confirm linearization by resolving 1–2 µL of the digest (50–100 ng of cut plasmid) by agarose gel electrophoresis with 50–100 ng of supercoiled (uncut) plasmid for comparison (see Note 19).
15. Ethanol precipitate the linearized plasmid: Add 3 M sodium acetate (pH 5.0–5.5) equal to 1/10 of the volume remaining after an aliquot has been removed for electrophoresis, and ice-cold 100% ethanol equal to twice the volume. (It is not necessary to purify the digestion reaction before precipitation). Vortex and incubate on ice for at least 10 min.
16. Centrifuge for 20 min at 4°C at 16,000 × *g*. Aspirate the supernatant fraction (see Note 20).
17. Add 750 µL of ice-cold 70% ethanol to the precipitated pellet, vortex, centrifuge as above, and aspirate the supernatant fraction. Briefly centrifuge again to collect residual ethanol and aspirate the supernatant fraction.
18. Dry the DNA pellet by leaving the tubes open for 5–15 min at RT in a clean, sterile environment, preferably a tissue-culture hood, until all liquid has evaporated. Resuspend the pellet in 5–10 µL of sterile water. Store on ice or at –20°C.

3.2. Transformation of *C. neoformans* by the pFrame Vector Using Electroporation

Proper sterile technique should be maintained throughout the procedure. Perform steps 3–8 on ice or at 4°C, as appropriate. Chill the electroporation buffer (EB), ultrapure water, 1.5-mL microfuge and 50-mL conical tubes, and the 0.2-cm electroporation cuvettes at 4°C before starting (see Note 21).

1. Inoculate a single colony (see Note 22) of a *ura5* mutant strain of *C. neoformans* from a freshly streaked agar plate (see Note 23) to 50 mL of YPD medium in a

¹⁸The exact amount depends on the strain of *C. neoformans* that will be transformed, but 2 µg are generally sufficient.

¹⁹A linearized plasmid will migrate as a discrete band at a rate proportional to its length, whereas the uncut supercoiled plasmid will generally migrate faster.

²⁰A pellet may or may not be visible on the inside wall of the tube. Care should be taken not to aspirate this pellet.

²¹Keeping the cells cold serves two purposes. First, the viability of the transformed cells is improved by preventing overheating due to the rapid dissipation of voltage during electroporation. Second, the pores induced in the membranes by the electrical pulse close more slowly at lower temperatures, allowing more time for DNA entry into the cells (48).

²²It is imperative to use a single colony to preserve the purity of the strain. If a single colony cannot be picked, we recommend to restreak the strain for isolation prior to this step.

²³It is best for the strain to have been restreaked onto fresh media within several days of the transformation and to have been maintained for no longer than 1–2 months. Optimal transformation efficiency can be achieved using a strain that has recently been revived from a glycerol stock.

250-mL Erlenmeyer flask. Incubate at 30°C with shaking at 230 rpm until the culture density is approximately $5\text{--}15 \times 10^7$ cells per mL (see Note 24).

2. Dilute the culture to 2×10^6 cells per mL in fresh YPD (use 50 mL per planned transformation) and grow as above until the culture density is between 6 and 10×10^6 cells per mL (see Note 25).
3. Transfer each culture to a 50-mL conical tube, pellet the cells by centrifugation for 5 min at 4°C, $2,000 \times g$, and decant the supernatant fraction.
4. Suspend each cell pellet in 5–10 mL of chilled ultrapure water and pool in a single 50-mL conical tube. Adjust the volume to 50 mL with chilled ultrapure water and centrifuge as above. Decant the supernatant fraction and repeat once more with 50 mL of chilled ultrapure water.
5. Suspend the washed cell pellet in 50 mL of chilled EB, add 200 μ L of 1 M DTT, invert several times to mix, and incubate on ice for 15 min (see Note 26). Centrifuge as above and decant the supernatant fraction.
6. Suspend the DTT-treated cell pellet in 50 mL of chilled EB, centrifuge as above, and decant the supernatant fraction.
7. Suspend the cell pellet in chilled EB to a final volume of 1–2 mL. Aliquot 3×10^8 cells per transformation to sterile 1.5-mL microfuge tubes, centrifuge for 1 min at 4°C, $2,000 \times g$, and aspirate the supernatant fraction.
8. Suspend each cell pellet in 20 μ L of chilled EB (see Note 27). Add 1–5 μ g of the linearized plasmid (see steps 14–18 in Subheading 3.1) in 5–10 μ L of sterile ultrapure water, flick the tube to mix, and transfer the cell suspension to a 0.2-cm electroporation cuvette. Repeat for any remaining samples. Include a sample with water alone as a negative control, and a sample with an unmodified pFrame as a positive control for transformation.
9. Electroporate each sample at 500 V, 25 μ F, 1,000 Ω (see Note 28), and then immediately add 0.5–1 mL of YPD (equilibrated to RT) to the cuvette and transfer the cell suspension to a 13-mL culture tube.
10. Shake the tubes for at 2 h at 30°C. Meanwhile, pre-warm uracil-deficient agar plates to 30°C (see Note 29).
11. Centrifuge the tubes for 1 min at RT, $2,000 \times g$. Aspirate most of the supernatant fraction, leaving ~200 μ L in which to suspend the cell pellet. Plate 20% and 80% of the cell suspension on the pre-warmed agar plates, and incubate at 30°C until colonies appear (see Note 30).

²⁴A more dilute or a more saturated culture will take longer to reach the desired density in the subsequent step. Also, the OD₆₀₀ may be used to determine the culture density if a suitable conversion factor has been empirically established for the specific strain.

²⁵Typically, this range is reached within 4–6 h. Transformation efficiency may decrease if the culture is allowed to grow for too long.

²⁶The DTT reduces disulfide bonds in the cell wall, thereby facilitating entry of the plasmid into the cells.

²⁷The cells should be suspended in as small a volume as possible to minimize the area of contact with the cuvette electrodes and thus increase the time needed for the voltage established across them to be dissipated, allowing the linear plasmid enough time to enter the cells (48).

²⁸Use as high a resistance as allowed by the apparatus. Samples that give a time constant longer than 20 ms usually result in a good yield of transformants.

²⁹It helps to dry the agar plates for 1–2 h before plating the transformants to better absorb the liquid cell suspension.

³⁰This usually takes 2–3 days.

3.3. Phenotypic Screening of Transformants Using the *ADE2* Sentinel of RNAi

1. Generate master plates (arrays of single colonies) from the transformation plates: Use sterile flat-tipped toothpicks to transfer individual colonies to fresh uracil-deficient agar plates, applying the cells onto 2–3-mm diameter spots in a pattern corresponding to the pins of a 48-pin replicator (see Note 31). Incubate at 30°C until the colonies have grown up sufficiently (i.e., 1–2 days).
2. Replica plate the uracil-deficient master plates onto low-adenine uracil-deficient agar: Sterilize a 48-pin replicator by passing the tips through a flame until red-hot and cool by pressing it into a sterile agar plate until sizzling stops. Gently touch the pin tips to the colonies on the master plate, and press against the surface of a fresh low-adenine uracil-deficient agar plate, moving the replicator slightly to generate 2–3-mm spots. Rinse the pins in 100% ethanol and repeat for any other master plates.
3. Incubate the agar plates at 30°C until a reddish color begins to develop in some of the clones, suggesting effective RNAi (see Note 32). Inspect the cellular and colony morphology of the clones for expected phenotypes. Any clone of interest should be restreaked for isolation on uracil-deficient agar prior to further characterization.

3.4. Extraction of Total RNA and Analysis of mRNA Abundance

TRIzol® reagent contains phenol and is therefore extremely hazardous. Everything that comes in contact with this reagent should be treated as hazardous waste and disposed of according to institutional guidelines. Use only certified RNase-free reagents, pipet tips, and tubes. Clean all equipment with 70% ethanol before starting, and change gloves frequently to avoid RNase contamination from human skin and dust.

1. For each clone of interest, inoculate a single colony from a freshly streaked uracil-deficient agar plate into 25 mL of medium in a 250-mL Erlenmeyer flask. Incubate at 30°C with shaking at 230 rpm until the culture density reaches $1\text{--}2 \times 10^8$ cells per mL.
2. For each culture, aliquot 3×10^8 cells to a 1.5-mL microfuge tube, centrifuge for 1 min at RT, $400 \times g$, and aspirate the supernatant fraction.
3. Suspend the cell pellet in 750 μ L of TRIzol® reagent and transfer to a 2-mL screw-cap tube on ice. Carefully add 750 μ L of glass beads, ensuring that no beads remain on the edge of the tube.
4. Bead-beat each sample 6–10 times using a mini beadbeater at 4°C, alternating 1 min beating with 2 min rests on ice (see Note 33).
5. Let the beads settle for a few seconds, then transfer the cell lysate to a new 1.5-mL microfuge tube on ice. Add 750 μ L of TRIzol® to the beads, mix and let the beads settle, and then pool the supernatant fraction with the lysate to obtain ~1 mL total cell homogenate.

³¹Alternatively, use the pattern of your choice in this step, and a replica block as described in Unit 1.3 of the Current Protocols in Molecular Biology series (49) for the next step.

³²The parent strain and an *ade2* mutant strain can be patched onto each low-adenine agar plate to better discriminate between the normal and pink colony colors.

³³The extent of lysis can be estimated by spotting 4 μ L of the cell suspension on a glass slide and viewing the cells under a microscope. Bead beating can be stopped when ~75% of the cells look broken or empty (i.e., either the cell wall is cracked or the inside of the cell is completely transparent). Alternatively, the use of zirconium silicate beads may give more efficient lysis.

6. Continue with the TRIzol® extraction as specified by the manufacturer. Store the total RNA at -80°C .
7. To compare the efficacy of RNAi in different clones, the mRNA abundance can be analyzed by the following methods:
  - a. Perform a Northern blot using the total RNA extracted from each clone as described in Unit 4.9 of the Current Protocols for Molecular Biology series (39);
  - b. Perform quantitative PCR (qPCR) using cDNA prepared from the total RNA (see Note 34) as described in the BIOS Advanced Methods series (40).

3.5. Preparation of the pIBB103 Vector for Triggering RNA Interference

1. Select a sequence to trigger RNAi following the methods given in Subheading 3.1, with the exception that the selected target sequence should not contain the recognition sites for the *SpeI* and *I-SceI* restriction enzymes (see Note 12).
2. Design a sense oligonucleotide primer that overlaps 20–25 bp of the 5' end of the selected target sequence and an antisense primer that overlaps 20–25 bp of the 3' end. Add an *SpeI* restriction site (ACTAGT) to the 5' end of each sequence, and then add six randomly arranged nucleotides to the 5' end of each resulting primer (see Note 13).
3. Amplify the selected target sequence by PCR using both primers in one reaction (see steps 5–7 in Subheading 3.1).
4. Digest 1 μg of the PCR product with 5–10 U of *SpeI* for 12–16 h at 37°C , and purify using a PCR purification kit. Store at -20°C .
5. Digest 1–2 μg of the pIBB103 plasmid with 5–10 U of *SpeI* for 12–16 h at 37°C , and purify using a PCR purification kit.
6. Ligate the *SpeI*-cut plasmid to the *SpeI*-cut target fragment and extract the fully assembled plasmid DNA (see steps 10–12 in Subheading 3.1). Store at -20°C .
7. Prepare the fully assembled plasmid for electroporation: See steps 14–18 in Subheading 3.1, with the exception that the completed vector should be digested with 10–15 U of *I-SceI*.

3.6. Transformation of *C. neoformans* by the Vector pIBB103 Using Electroporation

See Subheading 3.2, with the exception that, following electroporation, transformants should be selected on pre-warmed YPG agar plates containing 100 $\mu\text{g}/\text{mL}$ G418 (see Note 35).

3.7. Phenotypic Screening of Transformants Using the *URA5* Sentinel of RNAi

1. Pick single colonies from the transformation plates and transfer to YPG agar plates containing 100 $\mu\text{g}/\text{mL}$ G418. Incubate at 30°C until grown up sufficiently (see step 1 in Subheading 3.3).

³⁴To prepare cDNA, first remove contaminating DNA from the total RNA preparation using DNase, preferably with the Turbo DNA-free™ kit (Ambion). Alternatively, purify the mRNA using poly-T affinity chromatography using the Oligotex® mRNA mini kit (Qiagen). Next, generate cDNA from either the DNase-treated total RNA or purified mRNA using the SuperScript™ First-Strand Synthesis System for RT-PCR as per the manufacturer's instructions.

³⁵Growing the transformants on medium containing galactose induces the expression of the RNAi construct immediately after transformation. Alternatively, RNAi can be initially suppressed by selecting and maintaining the transformants on medium containing glucose (e.g., YPD). RNAi can then be induced by replica plating the clones onto galactose medium.

2. Replica plate the YPG plates onto 5-FOA agar plates containing galactose and G418 as well as onto uracil-deficient agar plates containing galactose and G418 (see step 2 in Subheading 3.3).
3. Incubate at 30°C until the transformants begin to grow on the 5-FOA medium. The same clones should not grow on uracil-deficient medium (see Note 36). Inspect the cellular and colony morphology of the clones for any phenotypes. Any clone of interest should be restreaked for isolation on YPG agar plates containing 100 µg/mL G418 prior to further characterization.
4. The efficacy of target gene down-regulation may be assessed by extracting total RNA and analyzing mRNA abundance as described in Subheading 3.4, with the exception that any clone of interest should be cultivated in YPG medium containing 100 µg/mL G418.

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³⁶Both the parent strain and a *ura5* mutant strain can be patched onto each agar plate to serve as controls for the growth phenotypes on both selective conditions. The parent strain should express a functional *URA5* gene and grow only on the uracil-deficient medium, whereas the *ura5* mutant strain should only grow on the 5-FOA medium. Since the efficacy of RNAi may differ between strains, it may take up to a week for the transformants to start growing on the 5-FOA.

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Fig. 1. RNAi mediated by episomally expressed hairpin RNA (hpRNA). **(a)** Schematic of an hpRNA-expressing construct, which consists of two antiparallel copies of a target sequence separated by a spacer region and placed between suitable promoter (P) and terminator (T) regions. **(b)** Transcription proceeds only along one strand to yield a single-stranded RNA molecule. **(c)** The complementary nature of the two antiparallel target sequences allows them to anneal intramolecularly to form a double-stranded duplex RNA that **(d)** is subject to cleavage by Dicer.

Fig. 2. RNAi mediated by convergent promoters. **(a)** Schematic of a converging promoter construct, which consists of a single copy of a target sequence flanked by promoter (P) and terminator (T) regions on each strand. **(b)** Transcription proceeds independently in both directions to yield single-stranded RNA molecules corresponding to the sense and antisense strands of the target sequence. **(c)** The individual transcripts anneal intermolecularly to form an RNA duplex that **(d)** is subject to cleavage by Dicer.

Fig. 3.

The pFrame vector mediates RNAi by means of a hairpin RNA (hpRNA). It allows the option of assessing the efficacy of RNAi by co-silencing endogenous *ADE2*. Constitutive synthesis of the hpRNA is regulated by an *ACT1* promoter (P_{ACT1}) and *GAL7* terminator (T_{GAL7}), and proceeds unidirectionally along a construct that consists of a fragment derived from *ADE2*, a fragment derived from *gusA*, a spacer derived from *GFP*, a second region derived from *gusA*, and the reverse complement of the same *ADE2* fragment. Insertion of a custom target fragment is accomplished by sequentially replacing each *gusA* piece.

Fig. 4.

The pIBB103 vector mediates RNAi by means of convergent promoters. It triggers co-silencing of endogenous *URA5*, enabling selection of clones undergoing interference by growth on medium containing 5-fluoroorotic acid. Transcription of both strands of the interfering construct is independently driven by converging, galactose-inducible *GAL7* promoters (P_{GAL7}) and terminators (T_{GAL7}). A custom target sequence can be inserted into the *SpeI* site. Digestion with the endonuclease *I-SceI* linearizes the plasmid and exposes telomeric repeats (*thick black bars*) that improve transformation efficiency.