

Published in final edited form as:

Methods Mol Biol. 2010 ; 638: 33–40. doi:10.1007/978-1-60761-611-5_3.

High-Throughput Construction of Gene Deletion Cassettes for Generation of *Neurospora crassa* Knockout Strains

Patrick D. Collopy, Hildur V. Colot, Gyungsoon Park, Carol Ringelberg, Christopher M. Crew, Katherine A. Borkovich, and Jay C. Dunlap

Abstract

The availability of complete genome sequences for a number of biologically important fungi has become an important resource for fungal research communities. However, the functions of many open reading frames (ORFs) identified through annotation of whole genome sequences have yet to be determined. The disruption of ORFs is a practical method for loss-of-function gene analyses in fungi that are amenable to transformation. Unfortunately, the construction of knockout cassettes using traditional digestion and ligation techniques can be difficult to implement in a high-throughput fashion. Knockout cassettes for all annotated ORFs in *Neurospora crassa* were constructed using yeast recombinational cloning. Here, we describe a high-throughput knockout cassette construction method that can be used with any fungal transformation system.

Keywords

Gene targeting; Filamentous fungi; *ku70*; *ku80*; *mus-51*; *mus-52*

1. Introduction

Many ORFs identified through whole-genome sequencing of numerous fungi cannot be functionally characterized based on sequence alone. Gene disruption serves as a fundamental approach for understanding the role of many ORFs that do not have an assigned function. We have undertaken the task of disrupting all ~10,000 ORFs in the annotated genome of *Neurospora crassa* as part of a functional genomics consortium (1). This project aims to provide fungal researchers with a set of individual gene knockouts that covers the whole *N. crassa* genome similar to the set of knockouts that encompasses the entire genome of the yeast *Saccharomyces cerevisiae* (2–4). While the scale of a whole-genome knockout project may not be feasible for all fungal researchers, systematic disruption of groups of functionally relevant ORFs can be quite informative. For example, the disruption of 103 putative transcription factors in *N. crassa* (5) demonstrated the usefulness of a high-throughput knockout procedure for fungi with completed genome sequences on a scale that is realistic for many suitably equipped labs.

One of the first hurdles to overcome with a large-scale knockout project like this is the construction of the knockout cassettes. The use of traditional restriction digestion and ligation, for example, is too cumbersome to be a favorable option for high-throughput knockout cassette assembly. For this reason, we employed a method that utilizes *S. cerevisiae* to assemble cassette components that possess short complementary overlapping sequence ends (6, 7). Components are synthesized in separate PCR reactions and cotransformed into *S. cerevisiae* for assembly by the host cell's own recombination machinery. An overview schematic for the construction of knockout cassettes using the yeast recombinational system is presented in Fig. 1.

Crucial to the success of this project was the work from Ninomiya et al. (8) demonstrating that mutation of single genes (*mus-51* and *mus-52*) involved in nonhomologous end-joining (NHEJ) leads to a high rate of homologous recombination in *Neurospora*. Prior to this work, high-throughput gene disruption in *N. crassa* was extremely difficult due to the low frequency of homologous recombination relative to ectopic insertions. We created two disruption strains, Δ *mus-51* and Δ *mus-52*, using the selectable marker *bar*, which confers resistance to phosphinothricin (9, 10). Knockout cassettes are transformed into one of the *mus* mutants to produce primary transformants, which are then crossed to wild type to generate knockout mutants in a genetic background lacking the *mus* mutation. Subsequent to our work, mutants deficient in the NHEJ-pathway have been shown to display high homologous recombination frequencies in a number of filamentous fungi (11). Therefore, assessment of targeting efficiency and generation of recipient strains that are deficient in the NHEJ-pathway are important areas of consideration prior to undertaking a large-scale gene deletion effort.

The basic protocol for the high-throughput construction of knockout cassettes in *N. crassa* genes is described here with some aspects of the procedure elaborated upon in greater detail. Additional details of the entire *N. crassa* process (including *N. crassa* electroporation and verification of targeted gene disruptions) can be found in the methods and Supplementary Methods of Colot et al. (5). Many of the protocols that were developed for *N. crassa* are easily transferred to other filamentous fungi. However, depending on the fungal organism of interest, *Agrobacterium*-mediated, biolistic or protoplast transformation methods may be more suitable than electroporation using this high-throughput knockout cassette construction protocol.

2. Materials

2.1. PCR of Knockout Cassette Components

1. *Gene-specific PCR products to be cloned.* Typically, these consist of three fragments: the 5' and 3' flanks of the gene(s) of interest and a resistance cassette. There should be approximately 30 bp of overlapping complementary sequence between fragments to be assembled. Four gene-specific primers are needed for each knockout cassette flank: 5' flank forward (5f), 5' flank reverse (5r), 3' flank forward (3f), and 3' flank reverse (3r). The following 5' common sequences were added to the respective primers for each gene to allow cassette assembly in the yeast recombinational system:
 - 5f, GTAACGCCAGGGTTTTCCCAGTCACGACG...
 - 5r, ATCCACTTAACGTTACTGAAATCTCCAAC...
 - 3f, CTCCTCAATATCATCTTCTGTCTCCGAC...
 - 3r, GCGGATAACAATTTACACAGGAAACAGC...
2. Plasmid pCSN44 (12, 13). This plasmid contains the dominant drug-resistance marker gene hygromycin B phosphotransferase (*hph*) from *E. coli*, flanked by the promoter and terminator of the *trpC* gene from *Aspergillus nidulans*.
3. Primers for the amplification of the *hph* resistance cassette: hphF (GTCGGAGACAGAAGATGATATTGAAGGAGC) hphR (GTTGGAGATTTGAGTAACGTTAAGTGGAT)
4. A high-fidelity, high-yield Taq polymerase to be used for the generation of gene flanks and resistance cassettes that minimizes errors in the final knockout cassettes. We use LA Taq by TaKaRa (Fisher Scientific, Pittsburgh, PA).

2.2. Yeast Transformation and Cassette Assembly

1. Cloning vector pRS426 (14). This is a 2 μ m vector that contains a *URA3* marker for selection in *S. cerevisiae*.
2. *S. cerevisiae* strain FY834 (*MAT α . his3 Δ 200 ura3-52 leu2 Δ 1 lys2 Δ 202 trp1 Δ 63*) (15).
3. *YPD medium*. Dissolve 10 g yeast extract, 20 g peptone and 20 g dextrose in dH₂O to a final volume of 1 L. Add 15 g agar and autoclave.
4. *96PEG solution*. Dissolve 45.6 g PEG (MW 3350) in dH₂O to a final volume of 88 mL. Add 10 mL 1 M lithium acetate and 2 mL 50 \times TE buffer. Autoclave or filter-sterilize.
5. *Sheared salmon sperm DNA, 10 mg/mL* (Fisher Scientific, Pittsburgh, PA). Dilute to 2 mg/mL in ddH₂O, boil for 5 min and store at -20°C . This does not need to be boiled each time it is thawed for use.
6. *SC-Ura medium*. To prepare 1 L, mix 26.7 g Drop-out Base with Glucose (US Biologicals, Swampscott, MA) and 2 g Drop-out Mix Synthetic Minus Uracil w/o Yeast Nitrogen Base (US Biologicals, Swampscott, MA) in H₂O. Autoclave.
7. Airpore tape sheets (Qiagen, Valencia, CA).

2.3. Yeast DNA Purification

1. Genra Puregene Tissue Kit (Qiagen, Valencia, CA).
2. Zymolyase T-100 (Seikagaku, Tokyo, Japan).
3. CleanSeq beads (Agencourt, Beverly, MA).
4. Agencourt SPRIPlate[®] 96R – Ring Magnet Plate (Agencourt, Beverly, MA).

3. Methods

Although many of the cassette construction steps were performed using a Biomek NX robot, these methods may also be easily scaled-down for small groups of genes.

3.1. PCR of Knockout Cassette Components

1. Gene-specific knockout cassettes should be designed to have 1–1.3 kb of gene-specific 5' and 3' flanks and a selectable marker (the hygromycin B phosphotransferase gene, *hph*, in our case). To ensure appropriate expression of the selectable marker, we use the *A. nidulans* promoter *trpC* (13).
2. For the primer design of 5' and 3' flanks, software written for us by John Jones (John Jones Consulting, Moreno Valley, CA) was used to retrieve regions adjacent to each ORF in the annotated genome of *N. crassa*. This information was then passed to PRIMER3 (<http://primer3.sourceforge.net/>), which would automatically select a list of candidate primers based on defined conditions (5).
3. Primers for the *Neurospora* knockout project are ordered from Illumina (San Diego, CA) in 96-well plates at 50 μM . These primers are mixed in three pairs (5f+5r, 3f+3r and 5f+3r) using a Beckman Biomek NX robot to a final concentration of 10 μM each. (The 5f+3r primer combination is required for the amplification of the final knockout cassette to be used for transformation.)
4. Perform separate 25 μL PCR reactions for the 5' and the 3' flanks using primer pair mixtures 5f+5r or 3f+3r. PCR reactions consist of: 2.5 μL 10 \times buffer (LA

Taq), 4 μL dNTP's (LA Taq), 1 μL 10 μM primer mixture, 0.25 μL genomic *N. crassa* DNA (prepared using the Puregene DNA kit; ~ 150 ng/ μL), 17 μL water, and 0.25 μL LA Taq (5 units/ μL).

5. Generate the *hph* cassette fragment in a 50 μL PCR reaction containing: 5 μL 10 \times buffer (LA Taq), 8 μL dNTP's (LA Taq), 1 μL 10 μM primer hphF, 1 μL 10 μM primer hphR, 0.5 μL pCSN44 DNA (from 1:100 dilution of plasmid miniprep), 34 μL water, and 0.5 μL LA Taq (5 units/ μL).
6. PCR cycling parameters for both flanks and the *hph* resistance marker are: 94°C for 1:00 min followed by 35 cycles of: {94°C for 30 s, 60°C for 30 s, 72°C for 2:00 min}, and a final extension of 72°C for 10:00 min.
7. Digest several μg of pRS426 vector with *EcoRI* and *XhoI* and dilute to a final concentration of 100 ng/ μL .

3.2. Yeast Transformation and Cassette Assembly

The PCR products and digested vector do not need to be cleaned up prior to yeast transformation. Yeast transformation was adapted from a 96-well protocol described in http://depts.washington.edu/sfelds/protocols/cloning_protocol.html

1. Inoculate 50 mL of YPD with 0.3 mL of a FY834 saturated culture. Grow overnight at 30°C with shaking (up to 300 rpm).
2. Pellet cells in a 50 mL conical tube (2,530 $\times g$ for 3 min). Discard supernatant, resuspend cells in 2 mL 100 mM lithium acetate and transfer to two microcentrifuge tubes.
3. Spin at top speed in a microcentrifuge for 30 s and discard the supernatant. Resuspend cell pellets in 100 mM lithium acetate to a total final volume of 1.8 mL (approximately 700 μL to each microcentrifuge tube). At this point, it is safe to keep cells on bench at room temperature until use.
4. Prepare fresh CT110 mixture (16). For 100 transformations mix 20.7 mL 96PEG, 580 μL boiled salmon sperm (2 mg/mL), 210 μL *hph* cassette (directly from PCR reaction of Section 3.1, step 5), 105 μL cut vector pRS426 (100 ng/ μL) and 2.62 mL DMSO. Mix thoroughly for 30 s, then add 1.8 mL yeast cells and mix thoroughly for an additional 60 s.
5. Pipette 200 μL of CT110 mixture into each well of a 96-well deep-well plate.
6. Add 4 μL of 5' flank and 4 μL of 3' flank PCR reaction for each gene. Seal plate, vortex 4 min, and incubate at 42°C for 30 min.
7. Spin plate at 2,000 $\times g$ for 7 min and aspirate off supernatant.
8. Add 200 μL SC-Ura to each well and resuspend by pipetting up and down.
9. Transfer 80 μL of resuspended transformed cells to 1 mL of SC-Ura liquid in each well of a 96-well deep-well plate. Seal the plate with Airpore tape (Qiagen) and grow 3 days at 30°C with shaking.

3.3. Yeast DNA Purification

The following steps are described as they were performed using the Biomek NX robot, with the exception of centrifugation and vortexing steps. The Genra Puregene™ Tissue kit (Qiagen) was used on the robot with a few modifications that are described below.

1. After 3 days, centrifuge the plate at 2,000 $\times g$ for 3 min.

2. Remove supernatant and resuspend pellet in 300 μL of Gentra cell suspension solution (Qiagen) containing 120 $\mu\text{g}/\text{mL}$ Zymolyase T-100 (Seikagaku).
3. Incubate the plate at 37°C for 40 min, then centrifuge at $830 \times g$ for 3 min.
4. Remove the supernatant and add 300 μL Gentra cell lysis solution (Qiagen) to the pellet and mix.
5. Add 100 μL Gentra protein precipitation solution (Qiagen) and mix.
6. Vortex the plate for 20 s and centrifuge at $3,070 \times g$ for 12 min.
7. Transfer 90 μL of supernatant into a round-bottomed 96-well plate, and add 10 μL CleanSeq beads and 143 μL fresh 85% ethanol. Mix components and incubate statically for 3 min.
8. Place the plate on an SPRI magnetic plate (Agencourt) for 10 min and aspirate the cleared solution.
9. With the plate remaining on the magnet, add 200 μL of 85% ethanol to each well. After 30 s, remove the ethanol and repeat this rinse with 200 μL of 85% ethanol.
10. Allow the plate to air-dry for 10 min and remove from the magnet.
11. Add 40 μL 1 \times TE to each well and mix.
12. Return the plate to the magnet for 5 min and elute DNA to a new 96-well plate.

3.4. PCR of Final Full-Length Knockout Cassette

1. 50 μL PCR reactions are performed to generate each deletion cassettes. PCR reactions consist of: 5 μL 10 \times buffer (LA Taq), 8 μL dNTP's (LA Taq), 2 μL 10 μM primer 5f+3r primer mixture, 4 μL yeast DNA, 30.5 μL water, and 0.5 μL LA Taq.
2. PCR cycling parameters for the final full-length knockout cassette is: 94°C for 1:00 min followed by 35 cycles of: {94°C for 30 s, 60°C for 30 s, 72°C for 5:00 min} and a final extension of 72°C for 10:00 min.
3. Estimate DNA yields by agarose gel electrophoresis.
4. At this point, the cassettes we use for creating the *Neurospora* Genome Project knockout strains are cleaned up with the Qiagen QIAquick 96 PCR Purification Kit #28181 according to the manufacturer's protocol prior to *N. crassa* transformation. 5 μL of purified PCR product usually yields a sufficient number of *N. crassa* transformants by electroporation, but this amount will vary depending on the recipient organism and the transformation technique.

Acknowledgments

This work was supported by grant P01 GM068087 from the National Institute of General Medical Sciences. We would like to thank John Jones for software design and LIMS implementation.

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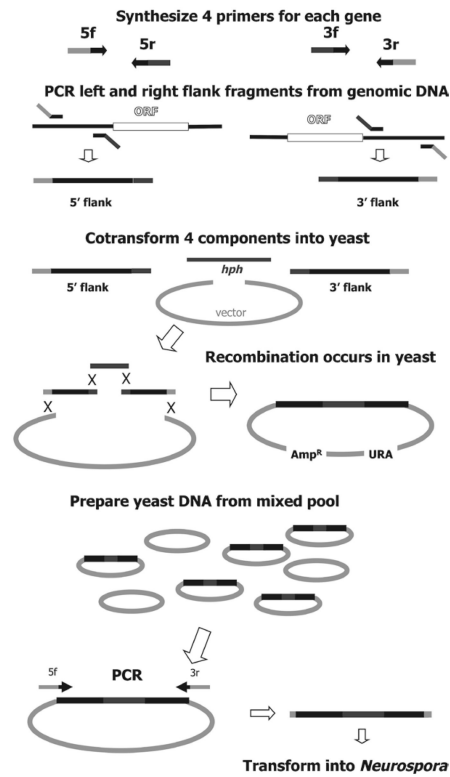


Fig. 1. Schematic of KO cassette construction using yeast recombinational cloning techniques. Primers were designed to amplify flanks both upstream and downstream of the ORF. Each primer also contained 29 nucleotides of “common” sequence to overlap with the yeast cloning vector (*light grey*) or the hygromycin B phosphotransferase (*hph*) resistance cassette (*dark grey*).