Published in final edited form as:
Methods Mol Biol. 2011 ; 722: 179–189. doi:10.1007/978-1-61779-040-9_13.

High-Throughput Production of Gene Replacement Mutants in *Neurospora crassa*

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Abstract

The model filamentous fungus *Neurospora crassa* has been the focus of functional genomics studies for the past several years. A high-throughput gene knockout procedure has been developed and used to generate mutants for more than two-thirds of the ~10,000 annotated *N. crassa* genes. Yeast recombinational cloning was incorporated as an efficient procedure to produce all knockout cassettes. *N. crassa* strains with the Δ*mus-51* or Δ*mus-52* deletion mutations were used as transformation recipients in order to reduce the incidence of ectopic integration and increase homologous recombination of knockout cassettes into the genome. A 96-well format was used for many steps of the procedure, including fungal transformation, isolation of homokaryons, and verification of mutants. In addition, development of software programs for primer design and restriction enzyme selection facilitated the high-throughput aspects of the overall protocol.

Keywords

Functional genomics; Gene deletion; High-throughput gene knockout; Large scale mutagenesis; Yeast recombinational cloning

1. Introduction

Genome sequences are now available for an increasing number of filamentous fungi. Recent advances in molecular-genetic approaches have accelerated identification of genes and genetic mechanisms governing pathogenesis and development in these organisms (1, 2). A collection of deletion mutants for all genes in the genome is a very useful resource for functional studies in all organisms, including filamentous fungi. In the yeast *Saccharomyces cerevisiae*, high-throughput production of gene deletion (also known as gene replacement or knockout) mutants was facilitated by the high rate of homologous recombination of DNA constructs (3). However, the low rate of homologous recombination observed in wild-type strains of most filamentous fungi limits the feasibility of such an approach.

Neurospora crassa is a filamentous fungus with ~10,000 predicted genes (4) and serves as a model for species that are important pathogens of plants and animals (5). There are several factors that support efficient production of gene knockouts in *N. crassa*: (1) the transformation efficiency is very high, requiring only a few micrograms of DNA (6), (2) transformation is easily accomplished by electroporation of conidia (7), and (3) the organism has a fast growth rate and is easy to culture (8). However, similar to other filamentous fungi, wild-type *N. crassa* strains have a very low rate of homologous recombination (<10%; (9)). This negative factor has been mitigated by a recent study demonstrating that deletion of

mus-51 or *mus-52*, genes required for nonhomologous end-joining DNA repair, greatly reduces ectopic integration and improves the incidence of homologous recombination (>90%; (10)).

The discovery that mutation of either *mus-51* or *mus-52* could produce a strain with levels of homologous recombination in excess of 90% provided a foundation for a high-throughput gene knockout method in *N. crassa*. In addition, the high recombination rate observed in *S. cerevisiae* was exploited to produce gene knockout cassettes for all *N. crassa* genes (11). Fragments corresponding to 1-kb fragments of DNA 5′ and 3′ to each open reading frame and the selectable marker hygromycin B phosphotransferase (*hph*) were amplified using the polymerase chain reaction, with primers designed to produce fragments with complementary ends. The fragments were then joined in vivo using yeast recombinational cloning (11) to produce the final gene replacement/knockout cassettes, with the 5′ and 3′ regions flanking the selectable marker *hph* (12).

In this chapter, we present a high-throughput procedure for production of gene knockout mutants in *N. crassa*. We first describe the protocol used for generation of *mus* deletion strains. The general method used to produce the Δ*mus* knockout cassettes is presented; for details regarding yeast strains, yeast media, etc., the reader is directed to another volume (20). It should also be noted that long flanking sequences (3 kb) were necessary to achieve homologous recombination of the Δ*mus* constructs in the wild-type background. In the last part of the chapter, we present the method for high-throughput production of knockout mutants for every gene in the genome, using the Δ*mus* mutants as transformation recipients. Again, details regarding construction of the actual knockout cassettes are presented in another volume (20).

2. Materials

Note: All plates are 100 mm in diameter and contain 30 ml agar medium.

2.1. Generation of N. crassa Δmus Mutants

1. *S. cerevisiae* strain FY834 (20).
2. YPD medium: in 100 ml, 1 g yeast extract, 2 g peptone, and 2 g dextrose. Autoclave.
3. SD-Ura medium: in 1 L, 6.8 g yeast nitrogen base, 20 g dextrose, and 2 g drop-out mix minus uracil (w/o yeast nitrogen base). Adjust pH to 5.8, add 15 g agar (for plates) and autoclave.
4. *N. crassa* strains FGSC 4200 (wild type, mat a) and FGSC 6103 (*his-3*, mat A).
5. Eppendorf electroporator 2510 (Eppendorf Scientific, Westbury, NY).
6. Electroporation cuvette with 2-mm gap width.
7. QiaexII gel extraction kit (Qiagen, Valencia, CA).
8. Phosphinothricin (PPT) purified as previously described (13).
9. 50× Vogel's minimal medium salts for use with PPT (50× VM-PPT salts; 1 L): 126.8 g $Na_3C_6H_5O_7 \cdot 2H_2O$, 250 g KH_2PO_4, 10 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCl_2 \cdot 2H_2O$, 5 ml biotin solution, 5 ml trace elements solution, and 5 ml chloroform as preservative (8).
10. Biotin solution (filter sterilize): 5 mg biotin/100 ml 50% (v/v) ethanol.

11. Trace elements (filter sterilize): in 100 ml, 5 g $C_6H_8O_7 \cdot H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 1 g $Fe(NH_4)_2(SO_4) \cdot 6H_2O$, 0.25 g $CuSO_4 \cdot 5H_2O$, 0.05 g $MnSO_4 \cdot H_2O$, 0.05 g H_3BO_3, and 0.05 g $NaMoO_4 \cdot 2H_2O$.
12. 10× FGS additive (filter sterilize): in 1 L, 5 g fructose, 5 g glucose, and 200 g sorbose (6).
13. VM-PPT medium: 1× VM-PPT Salts, 1.5% sucrose, 0.5% L-proline, and 1% agar (for solid medium) (14). Add PPT to 400 μg/ml after autoclaving.
14. VM-PPT-His medium: 1× VM-PPT salts, 1.5% sucrose, 0.5% L-proline, and 1% agar (for solid medium). Add PPT to 400 μg/ml and L-histidine to 100 μg/ml after autoclaving.
15. FGS-PPT plates: 1× VM-PPT salts, 0.5% L-proline, and 1% agar. After autoclaving, add 10× FGS additive to 1× final concentration and PPT to 400 μg/ml.
16. PPT regeneration agar: 1× VM-PPT Salts, 0.5% L-proline, 1 M sorbitol, and 1% agar. Add 10× FGS additive to 1× final concentration after autoclaving.
17. FGS-PPT-His plates: 1× VM-PPT Salts, 0.5% L-proline, and 1% agar. After autoclaving, add 10× FGS additive to 1× final concentration, PPT to 400 μg/ml, and L-histidine to 100 μg/ml.

2.2. High-Throughput Transformation of N. crassa

1. *N. crassa* strains FGSC 9718 (Δ*mus-51::bar*, *mat a*) and FGSC 9719 (Δ*mus-52::bar*, *mat a*).
2. 96-well 2 mm electroporation plate (BTX, Holliston, MA).
3. High-throughput electroporation plate handler (BTX).
4. Electro cell manipulator, model ECM 630 (BTX).
5. Sterile 96-deep-well plate (VWR, West Chester, VA).
6. Multichannel pipet (Rainin, Oakland, CA).
7. 15 ml EZ clip polyethylene tubes (Thermo Scientific, Waltham, MA).
8. Hygromycin B (Calbiochem, San Diego, CA).
9. 50× Vogel's salts (50× VM Salts; 1L): 126.8g $Na_3C_6H_5O_7 \cdot 2H_2O$, 250 g KH_2PO_4, 100 g NH_4NO_3, 10 g $MgSO_4 \cdot 7H_2O$, 5 g $CaCl_2 \cdot 2H_2O$, 5 ml Biotin solution, 5 ml Trace elements solution, and 5 ml chloroform as preservative (8).
10. Biotin solution (see above).
11. Trace elements (see above).
12. 10× FGS additive (see above).
13. VM: 1× VM salts, 1.5% sucrose, and 1% agar (for agar media) (8).
14. FGS-YE-His plates: 1× VM salts, 2% yeast extract, and 1% agar. After autoclaving, add FGS additive to 1×, L-histidine to 100 μg/ml, and hygromycin to 300 μg/ml.
15. His-YE Regeneration agar: 1× 50× VM Salts, 1 M sorbitol, 2% yeast extract, and 1% agar. Add FGS additive to 1× and L-histidine to 100 μg/ml after autoclaving.
16. Recovery medium: 1× VM salts, 2% yeast extract. Add L-histidine to 100 μg/ml after autoclaving.

2.3. Isolation of Homokaryotic Mutants

1. *N. crassa* strain FGSC 2489 (mat A), *fluffy* (*fl*) mutants FGSC 4317 (*mat A*) and FGSC4347 (*mat a*).
2. Synthetic crossing medium (SCM) agar: in 1 L, 1.0 g KNO_3, 0.7 g K_2HPO_4. 0.5 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, 0.1 g NaCl, 0.1 ml Biotin solution, 0.1 ml trace elements, 15 g sucrose, and 10 g agar. Autoclave to sterilize.
3. FGS-Hyg plates: 1× VM salts, 1% agar. Add FGS additive to 1× and hygromycin to 200 μg/ml after autoclaving.
4. VM-Hyg agar slants: 1× VM salts, 1% agar. Add hygromycin to 200 μg/ml final concentration after autoclaving.
5. VM-PPT agar (see above) in 12-tube strips: each tube contains 250 μl of VM-PPT agar medium.

2.4. Confirmation of Knockout Mutants by Southern Blot Analysis

1. 12-Well vacuum manifold (Millipore, Billerica, MA).
2. Whatman 2.5 cm grade 1 filter paper circles (Whatman, Piscataway, NJ).
3. 96-Deep-well plates (E&K Scientific, Santa Clara, CA).
4. Caps for 96-deep-well plates (Qiagen, Valencia, CA).
5. 5-mm stainless steel beads (Qiagen).
6. TissueLyser (Qiagen).
7. Puregene DNA extraction kit (Qiagen).
8. Qiagen MagAttract 96 DNA plant core kit (Qiagen).
9. PCR DIG probe synthesis kit (Roche, Basel, Switzerland).
10. DIG wash and block buffer set (Roche).
11. DIG Easy Hyb (Roche).
12. Anti-digoxigenin–AP conjugate (Roche).
13. DIG-labeled DNA molecular weight marker VII (Roche).
14. CDP-star (Roche).

3. Methods

3.1. Generation of N. crassa Δmus Mutants

3.1.1. mus Knockout Cassette Construction

1. PCR primers were designed to amplify the *bar* gene (15) and 3-kb fragments corresponding to the 5′ and 3′ regions of *mus-51* and *mus-52*. The 3-kb flanking regions are necessary to achieve a high rate of homologous recombination in a wild-type genetic background. The *bar* gene confers resistance to the chemical phosphinothricin, also known as Ignite or Finale.
2. Amplify the 3-kb 5′ and 3′ flanking regions of *mus-51* or *mus-52* and the *bar* gene using primers and genomic DNA (for *mus-51* and *mus-52* fragments) or plasmid pTJK1 (for *bar*; (16) as template.
3. Digest vector pRS426 with *Eco*RI and *Xho*I (see Note 1).

4. Purify all PCR products and linearized vector using the QiaexII gel extraction kit following the manufacturer's instructions.
5. Inoculate 50 ml of YPD with 0.3 ml of a saturated culture of yeast strain FY834 and incubate overnight at 30°C with shaking.
6. Pellet yeast cells in a 50-ml conical tube by centrifugation at 2,500 rpm for 5 min at room temperature.
7. Discard supernatant and resuspend cells in 0.4 ml 100 mM lithium acetate.
8. Transfer 50 μl of cells to a 1.5 ml microcentrifuge tube and spin down at maximum speed for 30 s at room temperature.
9. Completely remove supernatant and add the following ingredients in order: 240 μl 50% polyethylene glycol 3350, 36 μl 1 M lithium acetate, 50 μl boiled salmon sperm DNA (2 mg/ml), 27 μl sterile water, 1 μl linearized pRS426 (100 ng/μl), and 2 μl each of three PCR products.
10. After vortexing, incubate mixture at 42°C for 30 min.
11. Spin down cells as in step 8 and discard supernatant.
12. Resuspend cells in 1 ml YPD and recover by incubating at 30°C for 1 h.
13. Spin down cells at maximum speed for 30 s and discard supernatant.
14. Resuspend cells in 100 μl YPD and spread on SD-Ura agar plate.
15. After incubating at 30°C for 3–4 days, collect all yeast colonies by scraping the plate with a glass spreader and dipping in 1 ml of sterile water (see Note 2).
16. Spin down cells at maximum speed for 30 s and discard supernatant.
17. Extract yeast DNA using the "Smash and Grab" DNA extraction protocol (17).
18. *mus* knockout cassettes containing the *bar* gene flanked by 3-kb 5′ and 3′ regions of *mus-51* or *mus-52* are amplified using flanking primers with the extracted yeast DNA as template.

3.1.2. Transformation of N. crassa Wild Type with Δmus-51 or Δmus-52 Knockout Cassette DNA

1. Collect conidia of wild-type strain FGSC 4200 (*mat a*) from a 2-week-old culture and suspend in 50 ml of ice-cold sterile water.
2. Centrifuge the tube for 5 min at 2,500 rpm and discard the supernatant.
3. Resuspend conidia in 30 ml of sterile water by vortexing and repeat step 2.
4. Resuspend the conidial pellet in 30 ml of 1 M cold sorbitol.
5. Centrifuge the tube for 5 min at 2,500 rpm and discard the supernatant.
6. Repeat steps 4 and 5.
7. Resuspend the pelleted conidia in 1 M ice-cold sorbitol to give a concentration of 2.5×10^9 conidia/ml.

[1]Complete digestion is a critical step. Gel check on the digestion status is recommended.
[2]Normally, each plate will have 50–100 colonies. If too many colonies are formed on each plate, it is likely that the vector pRS426 is not digested completely.

8. Place 40 μl conidial suspension in a 1.5-ml sterile microfuge tube and add 1 μg *mus* knockout cassette DNA.
9. Transfer the mixture to a 2 mm electroporation cuvette and electropulse using an Eppendorf electroporator 2510 set at 2000 V (or other appropriate electroporator and settings; see below). Add 1 ml 1 M ice-cold sorbitol and transfer mixture to a new tube.
10. Combine the electroporation mixture with 10 ml PPT regeneration agar and plate on FGS-PPT plates.
11. Pick transformant colonies onto VM-PPT slants after incubation at 30°C for 3–4 days.

3.1.3. Verification of mus Knockout Mutants Using Southern Analysis

1. Inoculate 3 ml VM-PPT liquid cultures in 18 × 150 mm glass tubes using hyphae from the transformant slant cultures. Incubate with shaking at 30°C for 24 h. Collect cultures by vacuum filtration and place in 2-ml plastic microcentrifuge tubes.
2. Grind cells in liquid nitrogen using glass rods.
3. Extract genomic DNAs using the Qiagen Puregene DNA isolation kit according to the manufacturer's directions.
4. Digest DNA with an enzyme that will show a different banding pattern for the wild-type and gene replacement *mus* gene. Subject the digests to Southern blot analysis (18, 19), using the entire knockout cassette as a probe.

3.1.4. Isolation of Homokaryotic mus Knockout Mutants

1. We crossed heterokaryotic *mus* deletion mutants as males to strain FGSC 6103 (*his-3*, *mat* A). The *his-3* background was chosen to facilitate future experiments targeting constructs to the *his-3* locus of knockout mutants. However, the heterokaryons could just as easily have been crossed to a wild-type strain of opposite mating type, as was done for all knockout mutants created using the *mus* mutants as recipients (see Section 3.2.2, below).
2. Collect ascospore progeny and suspend in 1 ml of sterile water.
3. Centrifuge tubes at 3,000 rpm for 5 min at room temperature. Discard the supernatant.
4. Resuspend ascospores in 1 ml of sterile water. Withdraw 100 μl of suspension into a 1.5-ml microcentrifuge tube.
5. Incubate the tube at 60°C for 45 min to activate the ascospores.
6. Plate activated ascospores on FGS-PPT-His plates.
7. After 24 h incubation at 30°C in the dark, transfer germinated ascospores to VM-PPT-His agar slants.
8. Isolate genomic DNA from progeny for each gene as described above (section 3.1.3).
9. Perform Southern blot analysis as described above (section 3.1.3) to confirm homokaryotic knockout mutants of either *mus-51* or *mus-52*. The presence of the *his-3* mutation is determined by spot testing strains on VM, while mating type is determined using *fl* mating type tester strains (8). We selected Δ*mus-51 mat a*

strain FGSC9718 and Δ*mus-52 mat a* strain FGSC9719 as the transformation recipients for all knockout cassettes described in Section 3.2, below.

3.2. High-Throughput Gene Replacements in Δmus-51 or Δmus-52 Strains

3.2.1. Transformation of Δmus Strains with Knockout Cassettes

1. The knockout cassette for each target gene consists of the hygromycin phosphotransferase (*hph*) gene flanked by 1 kb 5′ and 3′ regions of the gene (12). The *hph* gene confers resistance to the antibiotic hygromycin (see Note 3).
2. Determine the linkage group of target genes from the genome sequence (http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7). In cases where the target and *mus* gene are on the same chromosome, the other *mus* mutant strain should be the recipient for transformation (see Note 4).
3. Cultivate the *mus* deletion strain in VM agar flasks for 3 days at 30°C in the dark, followed by 11 more days at 25°C in the light.
4. Collect conidia from the 2-week-old cultures and suspend in 50 ml ice-cold sterile water.
5. Wash the conidia twice using sterile water and then twice with 1 M ice-cold sorbitol, as described in Section 3.1.2, steps 2–6.
6. Resuspend the conidial pellet in 1 M sorbitol to yield a concentration of 2.5×10^9/ml.
7. Transfer the conidial suspension (40 μl/well) into 96-well electroporation plates on ice.
8. Add approximately 1 μg (5–10 μl) of knockout cassette DNA to each well using a multichannel pipet.
9. Electropulse the plate using a BTX ECM 630 electroporator set at 1,500 V, 600 Ω, and 25 μF.
10. Immediately add cold 1 M sorbitol (60 μl) to each well using a multichannel pipet.
11. Transfer the electroporation mixture to 900 μl of chilled 1 M sorbitol in a 96-deep-well plate on ice.
12. Transfer approximately 500–1,000 μl of the electroporation mixture to 1 ml of recovery medium (with 2% yeast extract and 100 μg/ml histidine) in a 96-deep-well plate and incubate at 30°C in the dark with shaking for 2 h (see Note 5).
13. After the recovery step, combine the mixture (1.5–2 ml) with 10 ml of His-YE regeneration agar in a 15-ml EZ clip poly-ethylene tube and spread on a FGS-YE-His plate.
14. Incubate plates at 30°C in the dark. Transformant colonies begin to appear after 4–7 days.
15. Pick four colonies/gene onto VM-Hyg agar slants.

[3]We recommend to use about 1 kb upstream and 1 kb downstream flanking sequences. Shorter flanking sequences may lead to lower efficiency of gene replacement.
[4]This will allow generation of knockout mutant progeny with wild-type copies of the *mus* alleles later in the protocol (section 2B).
[5]Addition of 2% yeast extract and 100 μg/ml histidine to recovery medium has previously been demonstrated to allow growth of various auxotrophic mutants (G.E. Turner, unpublished).

3.2.2. Generation of Homokaryotic Knockout Mutants with Wild-Type mus Alleles

1. Use two heterokaryotic transformants/gene as males to fertilize wild-type *mat A* strain FGSC 2489 cultured on SCM agar slants for 6 days at 25°C in constant light.
2. Incubate sexual crosses for 3 weeks at 25°C in constant light.
3. Collect and activate ascospores as described in Section 3.1.4, steps 2–5.
4. Plate activated ascospores on FGS-Hyg plates and incubate at 30°C for 1–2 days.
5. Pick 12 germinated ascospores from each plate onto VM-Hyg agar slants and incubate for 3 days at 30°C in the dark and then 2 days at 25°C in the light.
6. Check for the presence of the *mus* deletion by inoculating strains in 12-tube strips containing VM-PPT agar. Only those strains that do not grow on this medium (*mus*$^{+}$) are carried forward.
7. Determine the mating types of strains by crossing strains (male) to both mating types of *fl* tester strains (females; FGSC4317 *mat* A and FGSC4347 *mat* a).

3.2.3. Confirmation of Knockout Mutants by Southern Blot Analysis

1. Select two homokaryotic strains (one of each mating type) for each gene and use to inoculate 3 ml VM liquid cultures in 18 × 150 mm glass tubes.
2. Incubate tubes at 30°C in the dark with shaking for 2 days (longer for slow-growing mutants).
3. Collect tissues using a 12-well vacuum filter system. Transfer cell pads to 2-ml microcentrifuge tubes with screw caps or a 96-deep-well plate and store at −80°C.
4. Add liquid nitrogen to the tubes and pulverize the tissue using a glass rod. Alternatively, cells can be broken using a TissueLyser and 5-mm stainless steel beads.
5. Extract genomic DNA from ground tissues using the Qiagen MagAttract 96 DNA Plant Core Kit, following the manufacturer's recommendations.
6. Digest approximately 5 μg genomic DNA using an appropriate restriction enzyme. A program (http://borkovichlims.ucr.edu/southern/) was developed by John Jones for automatic identification of suitable restriction enzymes. Sequences of the wild-type (FGSC 4200) gene and the corresponding gene replaced with *hph* (as defined by the primers used to create the deletion cassette) were analyzed in silico with 17 selected restriction enzymes. The information was used to generate a list of usable enzymes and to report the sizes of the resulting bands that would hybridize to the probe (if the entire knockout cassette was used; see below) in both the wild-type and knockout strains.
7. Perform Southern blot analysis (18) using the entire knockout cassette or the *hph* gene as a probe. The DIG-labeled DNA molecular weight marker VII is run with samples on the agarose gel. The PCR DIG probe synthesis kit is used for probe amplification and labeling, while the DIG Easy Hyb, DIG wash and block buffer set, Anti-digoxigenin-AP conjugate, and CDP-Star are used for hybridization and detection.

Acknowledgments

This work was supported by National Institute of General Medical Sciences grant P01 GM068087. We thank John Jones for software design and implementation of the LIMS and Gloria Turner for helpful comments on the manuscript.

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