

How to create gene knockouts in *Neurospora*

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Background

Historically, it has been difficult to perform gene knockouts on a large scale in *Neurospora*, due to the low frequency of homologous recombination. For a functional genomics project, we developed a feasible high-throughput approach that has two significant components: 1) Yeast recombinational cloning was used to create the knockout cassettes; and 2) Novel *mus-51* and *mus-52* mutant strains based on the work of Inoue and coworkers were used as the recipients to virtually eliminate ectopic insertion of the knockout cassettes. The process of generating cassettes was performed on a pipetting robot but the essentials of the procedure were developed manually and are presented here. The overall scheme and the details for primer design are described in Colot and Park *et al.* The creation and characterization of the *Neurospora* knockout strains incorporate several modifications to existing techniques to facilitate handling of large numbers of strains, as well as an efficient and convenient method for the preparation of genomic DNA. We describe the high-throughput aspects and note variations suitable for small-scale use of the same procedures.

References

- Colot, H.V. and Park, G. *et al.*, A high-throughput gene knockout procedure for *Neurospora* reveals functions for multiple transcription factors. (2006) PNAS **103**, 10352-10357.
- Ninomiya, Y. *et al.*, Highly efficient gene replacements in *Neurospora* strains deficient for nonhomologous end-joining. (2004) PNAS **101**, 12248-12253.
- Dunlap, J.C. *et al.*, Enabling a community to dissect an organism: overview of the *Neurospora* functional genomics project. (2007) Adv. Gen. **57**, 49-96.
- Rose, M.D. *et al.*, (1990) Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Procedures

A. Generation of the knockout cassette DNA

Note: The overall scheme for cassette construction and the details needed for primer design can be found in Colot and Park, *et al.*, Fig. 1 and Supplementary Methods.

PCR flank fragments:

- do two separate 25 μ l reactions with gene-specific primer pairs 5f+5r or 3f+3r:

2.5 μ l 10x buffer (LA Taq)

4 μ l dNTPs (LA Taq)

0.5 μ l 10 μ M gene-specific primer 5f (or 3f)

0.5 μ l 10 μ M gene-specific primer 5r (or 3r)

0.25 μ l genomic DNA (e.g. Genra Puregene prep; \sim 150 ng/ μ l)

17 μ l water

0.25 μ l LA Taq (5 u/ μ l)

- LA Taq from TaKaRa, RR002M; order from Fisher: #TAKRR002M
- Puregene kit from Genra, #D-7000, now sold by Qiagen as #158667

PCR *hph* cassette:

5 μ l 10X buffer (LA Taq)

8 μ l dNTPs (LA Taq)

1 μ l 10 μ M primer *hph*F GTCGGAGACAGAAGATGATATTGAAGGAGC

1 μ l 10 μ M primer *hph*R GTTGGAGATTTTCAGTAACGTTAAGTGGAT

0.5 μ l pCSN44, diluted 1:100 from miniprep

34 μ l water

0.5 μ l LA Taq

N.B. the 5' ends of the *hph* primers are not complementary to pCSN44

Program for PCR:

94°C 1:00 min

35 cycles of :

 94°C, 30 sec

 60°C, 30 sec

 72°C, 2:00 min

72°C, 10:00 min

10°C, forever

Check yield of PCR products on a gel. With LA Taq you should get 100-500 ng/ μ l.

Digest pRS426:

- Digest miniprep vector DNA at a final concentration of 100 ng/μl with EcoRI and XhoI.
- The four components for yeast transformation (three PCR fragments plus cut vector) do not need to be cleaned up prior to transformation.

B. Yeast transformation and preparation of yeast DNA

Yeast transformation:

(based on <http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html>)

- grow a fresh 5-10 ml overnight culture of FY834 (inoculated from YPD plate) in YPD at 30°C
- the next day inoculate 50 ml YPD with 1 ml of overnight culture and grow at 30°C with shaking
- when O.D.₆₀₀=1 (or lower as long as cells have doubled twice), spin down cells in clinical centrifuge at top speed, 2-5 min
- rinse cells in 25 ml sterile H₂O & spin again
- decant H₂O, resuspend cells in 1 ml 100 mM lithium acetate; transfer to microfuge tube
- spin at top speed 15 sec; discard supernatant
- resuspend cell pellet in 400 μl 100 mM lithium acetate (if original O.D.=1; adjust volume if O.D. was different)
- keep on bench at rm. temp. till use

Then:

- vortex cells; pipette 50 μl into each empty transformation tube; spin 15 sec and completely remove supernatant
- to each cell pellet add 360-x μl transformation mix without fragments (where x is total volume of all PCR fragments, vector, etc.) – be careful, because mix is viscous – let tip drain completely
- add 100 ng of vector (1 μl) and 2 μl of each PCR reaction
- N.B.: these four components do not need to be cleaned up prior to yeast transformation

transformation mix, per sample:

240 μl 50% PEG 3350
36 μl 1 M lithium acetate
50 μl sheared salmon sperm DNA (2 mg/ml)
34-x μl sterile H₂O
x μl total volume of plasmid and PCR fragments.

- [if you don't make a master mix but add ingredients individually, add them in the order listed.]
- vortex each tube to completely suspend cell pellet
- incubate at 30°C for 30 min (this step has been eliminated from the Gietz lab protocol)
- mix by inverting; heat shock in a 42°C water bath for 30 min
- spin 15 sec; discard supernatant
- rinse cells in 1 ml sterile H₂O by gentle pipetting up and down; spin again
- remove all but 100-200 µl of medium; resuspend cells gently
- spread onto SC-Ura plates (e.g., with 10-20 sterile 4 mm glass beads)
- grow for 2-3 days at 30°C

You should get dozens or hundreds of colonies per plate. A negative control with vector alone will usually give several-fold fewer colonies than the experimental samples – this is due to uncut or rejoined vector and is not a problem.

Materials:

4 mm glass beads for plating: Fisher 11-312B – simply autoclave prior to use

sheared salmon sperm DNA: Fisher # E0032006957, 10mg/ml

- dilute to 2 mg/ml in H₂O
- boil 5 min and put immediately on ice
- store aliquots frozen and use repeatedly without reboiling

YPD:

6 g yeast extract
 12 g Peptone
 12 g dextrose
 (10 g agar for plates)
 600 ml H₂O

Autoclave (and pour plates).

SC-Ura plates:

26.7 g drop-out base with glucose (US Biologicals # D9500)
 2 g drop-out mix minus Ura (US Biologicals # D9535)
 15 g agar
 1 liter H₂O

Autoclave and pour.

50% PEG 3350:

- 50 g PEG 3350 (Sigma # P3640)
- add 35 ml H₂O and stir till dissolved (will take maybe 30 min)
- bring to 100 ml with H₂O
- filter sterilize (or autoclave)
- keep tightly capped; increasing concentration will lower efficiencies

“Smash-and-grab” yeast DNA preparation:

(based on Rose *et al.*)

- pipette 1-2 ml YPD onto transformation plate; scrape colonies off with the end of a microscope slide and pipette into a microfuge tube
- spin 15 sec; discard supernatant; if cell pellet is more than 50-75 µl, remove excess and discard
- to cell pellet add 0.2 ml lysis buffer, 0.2 ml phenol/CHCl₃ and 0.3 g 0.45-0.5 mm glass beads (e.g., use a calibrated scoop made from a cut microfuge tube pierced with a syringe needle)
- vortex 1-2 min
- spin 10 minutes; remove 100 µl supernatant to a fresh tube (avoid interface)
- add sodium acetate pH 5.2 to 0.3 M and 2.5 vol ethanol; mix
- spin 3-5 min; discard supernatant; rinse pellet with 70% ethanol and spin again
- air dry and resuspend pellet in 50 µl 1xTE

Note: if you want to purify the new KO cassette plasmids, or if your subsequent PCR result is poor, electroporate 1 µl of yeast DNA into *E. coli* and pick a few colonies to analyze. Plasmid DNA should be a successful template for PCR of the KO cassette (or it could be linearized and transformed into *Neurospora* directly).

Materials:

Smash-and-grab lysis buffer:

2% Triton X-100
1% SDS
100 mM NaCl
10 mM Tris pH 8.0
1 mM EDTA

0.5 mm glass beads: Biospec # 11079105 (<http://www.biospec.com/Beads.htm>)

- the manufacturer says that acid washing the beads is not necessary (contrary to traditional protocols); they recommend washing in 10% bleach for 5 minutes, followed by copious rinsing and baking to dry (autoclaving is optional)

PCR final full-length cassette:

5 µl 10X buffer (LA Taq)
 8 µl dNTPs (LA Taq)
 1 µl 10µM gene-specific primer 5f
 1 µl 10µM gene-specific primer 3r
 0.5 µl yeast DNA (try using less if PCR unsuccessful)
 34 µl water
 0.5 µl LA Taq

Program for PCR:

94°C, 1:00
 35 cycles of:
 94°C, 30 sec
 60°C, 30 sec
 72°C, 5:00 min
 72°C, 10:00 min
 10°C, forever

Optional: Clean up PCR product:

Use your favorite method and estimate concentration. The cassettes used for creating the *Neurospora* Genome Project knockout strains are cleaned up with the Qiagen MinElute 96-well system. However, up to 5 µl of most PCR products straight usually yields plenty of *Neurospora* transformants with no apparent ill effects.

C. *Neurospora* transformation, generation and genotyping of homokaryons, and verification of final strains.

Transformation:

Preparation of conidial suspension

1. Check linkage of the target gene to *mus-51* or *mus-52*; if there is linkage, select the other *mus* KO strain as the transformation recipient.
2. Subculture *mus-51::bar* (FGSC 9718) or *mus-52::bar* (FGSC 9719) on VM agar slants containing ignite (400 µg/ml). From subculture, inoculate a piece of mycelium onto 50 ml VM agar in a 250 ml Erlenmeyer flask (without ignite, for better growth). Incubate at 30°C for 3 days and then at 25°C in constant light for 7-10 additional days.

3. Harvest conidia from the flask using sterile water and transfer to a 50 ml conical tube.
4. Centrifuge for 2-5 min at 2500 rpm and carefully pour off water.
5. Resuspend conidial pellet in 15-20 ml sterile cold water with a pipette, avoiding mycelial debris on tube wall, transfer to a new tube, add water to 25 ml and centrifuge.
6. Wash conidial pellet twice using 25 ml cold sterile 1M sorbitol, with vortexing and centrifugation.
7. Resuspend conidia in 0.5 to 1 ml cold sorbitol and measure concentration by counting on a hemocytometer (or diluting and measuring OD₆₀₀; aim for a reading between 0.2 and 1.0). Then, adjust concentration to 2.5×10^9 conidia/ml (or OD₆₀₀ = 100) for electroporation.

Electroporation

1. For high-throughput transformation, place 40 µl conidial suspension into each well of a BTX 96 well electroporation plate (BTX #45-0450) on ice.
2. Add about 0.2 to 1 µg (5 to 10 µl) of knock-out cassette DNA to the conidial suspension.
3. Using the plate handler (BTX HT-100), electro-pulse each column of the plate using a BTX Electro Cell Manipulator (Model ECM 630) at 1500V, 600Ω, and 25µF.
4. After electro-pulsing the entire plate, immediately add 60 µl of cold 1M sorbitol to each well.
5. Transfer mixture to 900 µl of cold 1M sorbitol in a 96-well deep well plate. Take 100-200 µl of the mixture and add to 800 µl recovery solution in a 96-well deep well plate (e.g. Corning 3960).
6. Incubate the plate at 30°C with gentle shaking for 2-4 hr.
7. [For electroporating in single cuvettes, use the same settings, then add 1 ml cold 1 M sorbitol to the cuvette, and take 100-200 µl into recovery solution.]
8. Meanwhile, dispense 7 ml regeneration agar into 96 15-ml conical tubes and keep molten in a water bath at 50-60°C.
9. Transfer entire recovered electroporation mixture from one well to the regeneration agar in the conical tube and mix well.
10. Plate immediately on 20 ml FGS agar plates for transformation. Repeat with remaining wells and incubate plates at 30°C.
11. After 4-5 days, use sterile glass Pasteur pipettes to pick colonies onto VM agar slants containing hygromycin.

Crossing heterokaryotic KO mutants to wild type:

1. Inoculate wild type strain FGSC 2489 (mat A) as the female on 6 ml SCM agar in 18 x 150 mm glass tubes and incubate at 25°C in constant light for 6 days. You can see protoperithecia formed on the agar surface under a dissecting microscope.
2. Clean excess hyphae and conidia with a wet, sterile cotton swab (Fisher 14-959-90) from the tube wall facing the agar surface to create a clean surface for the

- shot ascospores.
3. Collect a moderate number of heterokaryotic mutant conidia from the VM+hyg slant using a wet cotton swab and suspend in 1 ml sterile water in a microcentrifuge tube.
 4. Dip cotton swab into the suspension and inoculate onto the 6-day-old female by gently rubbing or rolling onto the surface of the agar. Repeat once or twice.
 5. Incubate at 25°C in constant light. Perithecia should become visible within 4-6 days.
 6. Allow 3.5 weeks of incubation. You will see a black haze of ascospores on the surface of the glass tube opposite the agar surface.
 7. Collect ascospores by rubbing a wet cotton swab along the glass surface and then dipping into 1 ml sterile water in a microcentrifuge tube.
 8. Vortex and remove 30 µl into another microcentrifuge tube containing 120 µl sterile water. Save the remaining ascospore suspension at 4°C for reuse, if necessary.
 9. Heat-shock ascospores by placing the tube in a 60°C water bath for 30-45 min.
 10. Plate ascospore suspension on freshly-prepared FGS+hygromycin (200 µg/ml) plates and incubate 1-2 days, monitoring daily. [This can be done using a conventional spreader or with sterile 4 mm glass beads (Fisher #11-312B).]
 11. Using a dissecting scope, pick germinated ascospores from the plate onto 2 ml VM agar+hygromycin slants. We slice out the piece of agar surrounding the ascospore using flattened platinum wire. Incubate at 30°C for 1-2 days.
 12. Score germination rate.

Spot testing for ignite sensitivity and mating type:

1. Inoculate hygromycin-resistant homokaryotic strains onto VM+ignite agar (400-800 µg/ml) in a 96-well array of 12-tube strips (e.g., USA Scientific 1212-1610/1212-1200). Each 1.1 ml tube contains 200 µl VM+ignite agar. Optional: cover the entire array of tubes with a porous sticky membrane (USA Scientific 2920-1000). N.B.: the VM in this medium should not contain NH₄NO₃ – see below.
2. Incubate the tubes at 30°C for 2 days.
3. Score for growth and, based on the results, discard VM+hyg slants of ignite-resistant strains in order to eliminate homokaryotic KO strains that still have the *mus-51/52::bar* background.
4. Inoculate the ignite-sensitive strains onto plates of both mating types (A and a) of the *fl* mutant, previously cultured for 6-7 days at 25°C.
5. Incubate the plates at 25°C in constant light for 3-4 days and then score mating types.

Verification of strains by Southern blotting:

Collection of tissue for preparation of DNA

1. Select one strain from each mating type (or two primary heterokaryons, if

- homokaryons were not obtained) and inoculate 3 ml of liquid VM containing hygromycin (200 µg/ml). Incubate at 30°C with shaking for 1-2 days (or more, for slowly growing strains).
2. Collect the tissue using a vacuum filter setup, rinse with sterile water, and squeeze dry on filter paper.
 3. For high-throughput processing, place fresh tissue in either a 96-well array of strip tubes, as used for the ignite mini-slants (see above) or a 96-well deep-well plate (E&K Scientific; #661000). Add a 5 mm stainless steel bead (Qiagen #69989) to each well for grinding. [Alternatively, the tissue can be placed in individual 2 ml microfuge tubes, frozen, ground with a glass rod and processed with e.g. the Genra Puregene kit (#D-7000, now sold by Qiagen as #158667) using their recommendations for fungal tissue.]
 4. Cover the strips, if used, with strip caps; cover the plate, if used, with the corresponding capmat (E&K Scientific #401000) applied with a roller (E&K Scientific #EK-5000) (hint: the cap strips also fit the wells in these deep-well plates). Either place the plate at -80°C for later use or immediately freeze the plate in liquid nitrogen and then grind using a TissueLyser (Qiagen #85210) at 30 Hz for 2 x 1 min. Between these two steps, switch the orientation of the plate/tubes as recommended and refreeze in liquid nitrogen for 30 sec.

Genomic DNA extraction

We use the Qiagen MagAttract 96 DNA Plant Core Kit (#67161) for high-throughput genomic DNA preps. The required magnetic plate is not included in the kit and must be purchased separately (#36915). We do this procedure manually, not on a robot, and we follow their recommended protocol except: use 400 µl RLT buffer and before adding it to the ground tissue, warm the plate in a -20°C freezer to allow easy removal of the cap strips or capmat. Then add RLT immediately at room temperature, recap (perhaps with fresh caps or mat), shake and vortex to lyse and suspend tissue thoroughly, and spin at 4,000 rpm for 10-20 min. For the final elution step use 50 µl of Buffer AE.

Gel running and Southern blotting

Follow standard molecular biology protocols for pouring, running and transferring the gel.

Digestion and probing of genomic DNA

1. We verify each KO strain by hybridization with a probe made either from the entire deletion cassette or from only the *hph* portion. Recommended enzymes and resulting diagnostic fragments for all KO strains made with our cassettes can be determined by going to <http://borkovichlims.ucr.edu/southern/> and entering the NCU number as NCUxxxxx.2.
2. We make probes with the Roche PCR DIG kit (#11636090910). We are not including the entire protocol for this here, assuming that most labs have an established method. However, we follow Roche's labeling and detection protocols without significant modifications.

Media and Solutions

The recipes for 50x Vogel's Medium, 10x FGS, biotin, SCM and trace elements are readily available. We have given details for media and solutions that may differ from standard recipes.

1. <u>VM agar</u>		<u>VM agar for ignite</u>	
<u>per 100 ml:</u>		<u>per 100 ml:</u>	
50x Vogel's	2 ml	50x Vogel's <u>without NH₄NO₃</u>	2 ml
sucrose	1.5 g	sucrose	1.5 g
agar	1 g	L-proline	0.5 g
H ₂ O	98 ml	agar	1 g
		H ₂ O	98 ml

Autoclave; cool to 60°C and add selection reagent (hygromycin to 200 µg/ml, ignite to 400 µg/ml).

2. FGS/yeast extract/His/hyg for plating transformations

<u>per liter:</u>	
50x Vogel's	20 ml
yeast extract	20 g
agar	10 g
H ₂ O	878 ml

Autoclave; cool to 60°C and add 100 ml 10x FGS, 2 ml 500x L-histidine (100 µg/ml final), and hygromycin (to **300 µg/ml**).

3. FGS+hyg plates for germinating ascospores

<u>per 100 ml:</u>	
50x Vogel's	2 ml
agar	1 g
H ₂ O	88 ml

Autoclave; cool to 60°C and add 10 ml 10x FGS and hyg to 200 µg/ml. Make fresh; plates more than 1 week old do not give consistent results.

4. Regeneration agar

<u>per 600 ml:</u>	
50x Vogel's	12 ml
sorbitol	109.2 g
yeast extract	12 g
agar	6 g
H ₂ O	504 ml

Autoclave; add 60 ml of 10x FGS and 1.2ml of 500x L-histidine

5. **Recovery solution**

per 600 ml:

50x Vogel's	12 ml
yeast extract	12 g
H ₂ O	587 ml

Autoclave; add 1.2 ml 500X L-histidine.

6. **500x L-histidine (50 mg/ml)**

per 100 ml:

L-histidine	5 g
H ₂ O to 100 ml	

7. **Ignite stock**

- Purchase concentrated Finale at a garden supply store. Ignite (glufosinate-ammonium) is the active ingredient at 5.78% (this value may vary).
- Shake 100 ml with 100 ml 1-butanol in a separatory funnel, let the aqueous phase (bottom) separate completely and then collect it. The volume will have decreased.
- Re-extract the aqueous phase with an equal volume of 1-butanol, let separate completely and collect again.
- Pour into a 250 ml bottle and add 10 ml of activated charcoal (Sigma #C7606) – this is a fine powder and can be measured in a 15-ml conical tube. Shake vigorously by hand for a few minutes.
- Filter out the charcoal using Whatman #1 filter paper. The solution will now be nearly colorless and will still have the odor of butanol.
- Assume 100% recovery of the starting amount of 5.78 g and calculate the approximate concentration.
- Test the growth of wild-type and *bar* strains on VM agar with increasing concentrations of ignite, from 200 to 1200 µg/ml. Be sure to use VM lacking NH₄NO₃. There is no need to filter-sterilize the stock.
- ~400 µg/ml is usually optimal for selection of transformants (i.e., growth of wild-type is largely inhibited but *bar* strain growth is not severely affected), but higher concentrations (e.g. 800 µg/ml) are more reliable for screening knockouts in the mini-tube format for the presence or absence of *bar* in the background.