How to create gene knockouts in *Neurospora*

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This document will present protocols and guidelines for making your own knockout strains of *Neurospora*. In order to achieve high-throughput production of knockout strains as part of the Program Project, we have created novel procedures and software tools, as well as adapting, simplifying and streamlining existing techniques.

We will briefly describe the overall scheme and then elaborate on certain details, including primer design, yeast recombinational cloning for assembling the deletion cassettes, the use of magnetic beads for isolation of yeast DNA, the creation of *mus-51* and *mus-52* strains, 96-well electroporation into *Neurospora*, a modified transformation medium, mini-slants for spot-testing, the use of magnetic beads for *Neurospora* DNA preps, and a custom-written application for designing Southern blots.

We have performed significant portions of the work on a pipetting robot. However, the protocols were first developed manually and can be done without the robot with small numbers of samples or in a 96-well format. We will provide protocols for performing all the procedures without the need for any specialized equipment, along with information on more expensive options suitable for high throughput.

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Note that there are several relevant web-based resources, including lists of primers used, lists of knockout strains completed, updated protocols, and the programs for both primer design and Southern design. The web sites include:

http://www.dartmouth.edu/~neurosporagenome/primers.html
http://www.dartmouth.edu/~neurosporagenome/protocols.html
http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html
http://borkovichlims.ucr.edu/southern/
http://borkovichlims.ucr.edu/primer/primerDesign.py

PCR flank fragments:

- do 2 separate 25 µl reactions with gene-specific primer pairs 5f+5r or 3f+3r:

2.5 µl 10x buffer (LA Taq)
4 µl dNTPs (LA Taq)
0.5 µl 10 µM gene-specific primer 5f (or 3f)
0.5 µl 10 µM gene-specific primer 5r (or 3r)
0.25 µl genomic DNA (Gentra Puregene prep; ~ 150 ng/ul)
17 µl water
0.25 µl LA Taq (5 u/µl)

- LA Taq, by TaKaRa; order from Fisher: cat. # TAKRR002M 250 units ca. $270
- PureGene kit from Gentra (www.gentra.com) cat. # D-7000A

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 ca. 1 kb PCR products by comparison to known fragments – you should get 200-500 ng/µl

PCR hph cassette

5 µl 10X buffer (LA Taq)
8 µl dNTP's (LA Taq)
1 µl 10uM primer hphF GTCGGAGACAGAAGATGATATTGAAGGAGC
1 µl 10uM primer hphR GTTGGAGATTTCAGTAACGTTAAGTGGAT
0.5 µl pCSN44, diluted 1:100 from miniprep
34 µl water
0.5 µl LA Taq

Program for PCR:
    same as for 1 kb flanks above
    (check yield as above; similar amount expected)

Digest pRS426 with EcoRI and XhoI:

Cut several µg of miniprepped vector DNA at a final concentraton of 100ng/µl

The four components for yeast transformation (three PCR fragments plus cut vector) <u>do not</u> need to be cleaned up prior to transformation.

One version of yeast transformation and DNA prep protocol:

(we describe two tested versions of these procedures; this one involves an inefficient transformation procedure followed by selection in liquid, not plates, and is the manual version of the one we developed for high-throughput cassette creation, suitable for our pipetting robot)

[adapted from the 96-well transformation protocol we use, which is modified from: http://depts.washington.edu/sfields/protocols/cloning_protocol.html]

Make 96PEG:

45.6 g PEG 3350
Dissolved in H_2O up to a final volume of 88 ml
10 ml 1M lithium acetate (LiOAc)
2 ml 50X TE

autoclave (or filter sterilize)

- Inoculate 50 ml YPD (see below) in a 250 ml erlenmeyer flask with 0.3 ml of a saturated FY834 culture. Grow overnight at 30 degrees with shaking (up to 300 rpm).
- Spin out cells in a 50 ml conical tube (3500rpm, 3 min), pour off supernatant, add 2ml 0.1M LiOAc and resuspend. Transfer to two microfuge tubes.
- Spin 30 sec at top speed and resuspend cells to a final volume of 1.8 ml 0.1M LiOAc (add about 700 µl to each microfuge tube).
- Meanwhile make "CT110" in a 50 ml conical tube…

The four components for yeast transformation (three PCR fragments plus cut vector) do not need to be cleaned up prior to transformation.

CT110 (for 10 transformations):

2.07 ml 96PEG
58 µl boiled salmon sperm DNA (2mg/ml)
21 µl hph cassette (PCR reaction, straight)
10.5 µl cut vector pRS426 (100ng/µl, straight)
0.262 ml DMSO (add last and mix hard for 30 sec.)
180 µl yeast cells (add after DMSO and mix hard by hand for 1 min.)

- Pipet 200 µl CT110 into each microfuge tube
- Add 4 µl 5' flank and 4 ul 3' flank PCR reaction for each gene (straight)
- Vortex 4 min
- Incubate at 42° for 30 min
- Spin 15 sec at top speed
- Aspirate off supernatant
- Add 200 µl SC-Ura and resuspend by pipetting up and down
- Add 80 µl of resuspended transformed cells to 1ml of SC-Ura liquid in a 5 ml sterile round-bottomed tube (e.g., Falcon 2059)
- Grow 3 days at 30° with shaking

YPD: for 600ml (or 20 plates):

6 g yeast extract
12 g peptone
12 g dextrose
(10 g Agar)
600 ml water
Autoclave
(pour plates)

SC-Ura:

purchase the following items from USBiologicals http://www.usbio.net:
*D9500 Drop-out Base With Glucose (Powder) 100g
*D9535 Drop-out Mix Synthetic Minus Uracil w/o Yeast Nitrogen Base (Powder) 25g

for a liter of liquid medium:

26.7 g of the drop-out base with glucose
2 g drop-out mix minus Ura

autoclave (or filter sterilize)

(if you are familiar with and have access to more traditional reagents for this medium, that is also fine)

eppendorf sheared salmon sperm DNA:

Fisher cat. # E0032006957 (10x1 ml @10mg/ml)

- dilute to 2 mg/ml in water
- boil it for 5 min and plunk into ice

(doesn't need to be reboiled each time you thaw it)

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Prepare yeast DNA with the Gentra yeast DNA kit:

(the following is slightly modified from their protocol:
http://www.gentra.com/pdf/01160.pdf)

the solutions are available from Gentra as Puregene kit D-6000A or separately; if you have their Puregene solutions for Neurospora DNA preps, you only need to order the Cell Suspension Solution (D-6001) and Lytic Enzyme Solution (D-6002)

PUREGENE DNA Purification Kit
DNA Purification From 1 ml Yeast Culture Medium

Cell Lysis

1. Add 1 ml cell suspension (*e.g.*, overnight culture) to a 1.5 ml tube on ice.
2. Centrifuge at 13,000-16,000 x g for 5 seconds to pellet cells and remove supernatant.
3. Add 300 µl **Cell Suspension Solution** to cell pellet and gently pipet up and down until cells are suspended.
4. Add 1.5 µl **Lytic Enzyme Solution** and invert tube 25 times to mix. [On the robot we are using Zymolyase; details available on request: knockouts@dartmouth.edu]
5. Incubate at 37°C for 30 minutes to digest cell walls. Invert sample occasionally during the incubation.
6. Centrifuge at 13,000-16,000 x g for 1 minute to pellet the cells. Remove supernatant.
7. Add 300 µl **Cell Lysis Solution** to the cell pellet and gently pipet up and down to lyse the cells.

Protein Precipitation

1. Add 100 µl **Protein Precipitation Solution** to the cell lysate.
2. Vortex vigorously at high speed for 20 seconds to mix the **Protein Precipitation Solution** uniformly with the cell lysate.
3. Centrifuge at 13,000-16,000 x g for 3 minutes. The precipitated proteins will form a tight white pellet. If the protein pellet is not visible, repeat Step 2 followed by incubation on ice for 5 minutes, then repeat Step 3.

[N.B. this is the point in the robot protocol at which we take the supernatant and purify DNA from it with Agencourt's CleanSEQ magnetic beads]

DNA Precipitation

1. Pour the supernatant containing the DNA (leaving behind the precipitated protein pellet) into a 1.5 ml microfuge tube containing 300 µl **100% Isopropanol**
2. Mix the sample by inverting gently 50 times.
3. Centrifuge at 13,000-16,000 x g for 1 minute; the DNA might be visible as a small white pellet but don't worry if you don't see a pellet.
4. Pour off supernatant and drain tube briefly on clean absorbent paper. Add 300 µl **70% Ethanol** and invert tube several times to wash the DNA pellet.
5. Centrifuge at 13,000-16,000 x g for 1 minute. Carefully pour off the ethanol.
6. Invert and drain the tube on clean absorbent paper and allow to air dry 10-15 minutes.

DNA Hydration

1. Add 50 µl **DNA Hydration Solution** (50 µl will give a concentration in the range of 100 to 200 ng/µl). (we do not do the recommended Rnase treatment at this stage)
2. Rehydrate DNA by incubating sample 1 hour at 65ºC and/or overnight at room temperature. If possible, tap tube periodically to aid in dispersing the DNA.
3. Store DNA at 4°C. For long-term storage, store at -20°C or -80°C.

PCR final full-length cassette:

5 µl 10X buffer (LA Taq)
8 µl dNTP's (LA Taq)
1 µl 10uM gene-specific primer 5f
1 µl 10uM gene-specific primer 3r
4 µl yeast DNA
30.5 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

by your favorite method and estimate yield. In our lab we don't always clean it up – 5 µl of most PCR products straight plus 5 µl water usually gives plenty of *Neurospora* transformants in the *mus-51/52* deletion strains with no apparent ill effects. However, the cassettes we use for creating the Neurospora Genome Project knockout strains are cleaned up with the Qiagen QIAquick 96 PCR Purification Kit #28181.

Several hundred ng PCR fragment in a total volume of up to 10 µl should be sufficient – if you are using non-cleaned product, it is wise to transform several amounts.

Another version of yeast transformation and DNA prep protocol:

(we describe two tested versions of these procedures; this one involves a very efficient transformation procedure, followed by selection on plates and a quick "smash-and-grab" DNA prep. This works well in our lab but some labs have problems with the final PCR and resort to transforming E.coli, as noted)

Yeast transformation (based on protocol from Gietz lab):

http://www.umanitoba.ca/faculties/medicine/biochem/gietz/Trafo.html

- grow up 5-10 ml fresh overnight of FY834 (inoculated from YPD plate) in YPD at 30°C.
- in a.m., inoculate 50 ml YPD with 1 ml of overnight and grow again at 30°C., with shaking
- check $O.D._{600}$ after 4 hr; aiming for O.D.=1 (lower is okay as long as cells have doubled twice) and it might take at least 5 or 6 hr
- spin down cells in clinical centrifuge – top speed, 2-5 min
- rinse cells in 25 ml sterile H_2O & spin again
- decant H_2O and suspend cells in 1 ml 100 mM LiOAc; transfer to microfuge tube
- spin at top speed 15 sec; pipette off LiOAc
- resuspend cells in 400 µl 100 mM LiOAc (if O.D.=1; adjust volume if O.D. different)
- keep on bench at rm. temp. till use

Then:

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

transformation mix, per sample:

240 µl 50% PEG 3350* (w/v)
36 µl 1 M lithium acetate (LiOAc)
50 µl carrier DNA* (see below for preparation of this)
34-x µl sterile H_2O
x µl total vol of plasmid and PCR fragments.

- [OR 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. (I use air incubator) for 30 min (this step has been eliminated from the Gietz lab protocol but I haven't yet done it that way)
- mix by inverting; heat shock in water bath for 30 min at 42 °C.
- spin down cells 15 sec; pipette off transformation mix

- rinse cells in 1 ml sterile water by gentle pipetting; spin again
- flick off all but 100-200 µl of medium; resuspend cells gently
- plate on SC-Ura plates* (I use sterile 4mm glass beads* for plating)
- grow for 2-3 days at 30°C.

what to expect: you should get dozens or perhaps hundreds of colonies per plate

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

*eppendorf sheared salmon sperm DNA: (see previous yeast protocol)

YPD: for 600 ml: (see previous yeast protocol)

SC-Ura plates:

purchase these items from USBiologicals http://www.usbio.net:

*D9500 Drop-out Base With Glucose (Powder) 100g
*D9535 Drop-out Mix Synthetic Minus Uracil w/o Yeast Nitrogen Base (Powder) 25g

for a liter of plates:

26.7 g of the drop-out base with glucose
2 g drop-out mix minus Ura
15 g agar
autoclave and pour

50% PEG 3350:

- 50 g PEG 3350 (Sigma P 3640)
- add 35 ml water and stir till dissolved (will take maybe 30 min)
- bring to 100 ml with water
- filter sterilize (or autoclave)
- keep tightly capped; increasing concentration will lower efficiencies

Yeast "smash-and-grab" DNA prep:

- pipette 1-2 ml YPD onto transformation plate; scrape colonies off with the end of a glass slide and pipette into a microfuge tube
- spin down 15 sec; pipette off sup; 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/$CHCl_3$ and 0.3 g 0.45-0.5 mm glass beads* (I use calibrated scoop made from cut microfuge tube pierced with syringe needle) – seal carefully because beads can cause sealing problem at rim
- vortex 1-2 min; wear gloves!
- spin 10 minutes; remove 100 µl supernatant to fresh tube (don't go too close to interface)

- to supernatant, add NaOAc to 0.3 M and 2.5 vol ethanol
- spin 3-5 min; discard supernatant; rinse pellet with 70% EtOH and spin again
- air dry and resuspend pellet in 50 µl 1xTE

..there will be some background colonies due to end-joining or uncut vector but it is not a problem

..if you want to purify the new KO plasmids, or if your PCR result is poor, electroporate 1 µl of this DNA prep into E. coli and pick 6 or so to analyze (chemical transformation didn't work for me)

smash-and-grab lysis buffer:

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

[based on M.D. Rose, F. Winston, and P. Hieter (1990) Methods in Yeast Genetics: A Laboratory Course Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.]

*0.5 mm glass beads: the cheapest source I know of is Biospec (http://www.biospec.com/Beads.htm) Cat. No**. 11079105** 454g

-- they say that acid washing is not necessary (contrary to traditional protocols) – the recommended protocol is washing in 10% bleach for 5 minutes, followed by copious rinsing and baking to dry (autoclaving is optional)
(-- I don't know that it's essential to preclean brand new beads before using them – I suspect it isn't)

PCR final full-length cassette: (see previous yeast protocol)

Neurospora transformation, 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 the *mus* knock-out strain on VM agar slants containing ignite (400 μg/ml). From subculture, inoculate a piece of mycelium onto VM agar in a 250ml Erlenmeyer flask (without ignite for better growth). Incubate at 30˚C for 3 days and then at 25˚C 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 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 30 ml and centrifuge.
6. Wash conidial pellet using 30 ml cold sterile 1M sorbitol twice, 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 OD_{600}; aim for a reading between 0.2 and 1.0). Then, adjust concentration to 2.5×10^9 conidia/ml (or $OD_{600} = 100$) for electroporation.

Electroporation

1. Place 40μl conidial suspension into each well of a BTX 96 well electroporation plate (BTX #45-0450) on ice.
2. Add about 0.5 to 1 μg (5 to 10 μl) of knock-out cassette DNA to the conidial suspension using a multichannel pipette.
3. Put the electroporation plate on the plate handler (BTX HT-100). Electro-pulse each column of the plate using 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 using a multichannel pipette.
5. Transfer mixture to 900 μl of cold 1M sorbitol in a 96-well deep well plate using

a multichannel pipette.

6. Take 100-200 µl of the mixture and add to 1ml recovery solution in a 96-well deep well plate (e.g. Corning 3960) using a multichannel pipette.
7. Incubate the plate at 30˚C with gentle shaking for 2-4 hr.
8. Meanwhile, dispense 10 ml regeneration agar into 96 15 ml conical tubes using a Wheaton Omnispense and keep molten in a water bath at 50-60˚C.
9. Transfer entire recovered electroporation mixture from each well to the regeneration agar in the conical tube and mix well.
10. Plate immediately on FGS agar plates (100mm) containing yeast extract, His and hygromycin (**300 µg/ml**) and incubate at 30˚C.
11. After 4-5 days, use sterile glass Pasteur pipettes to pick colonies onto VM agar slants containing hygromycin (200 µg/ml).

Crossing heterokaryotic KO mutants to wild type

1. Inoculate wild type strain FGSC 2489 (matA) 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 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 suspension and inoculate onto the 6-day-old female by gently rubbing or rolling on 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 (not less!). 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 150 µl into another microcentrifuge tube. 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. (**don't go longer than 45 min!**)
10. Plate ascospore suspension on FGS+hygromycin (200 µg/ml) plates and incubate. (This can be done using a conventional spreader or with sterile 4 mm glass beads (Fisher #11-312B).)
11. After 1-2 day incubation, pick 12 germinated ascospores from the plate onto 2 ml VM agar+hygromycin (200 µg/ml) 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 test for ignite sensitivity and mating type (in 96 well format)

1. Inoculate a piece of mycelium onto VM+ignite agar (400 µg/ml) in a 96-well array of 12-tube strips (e.g., USA Scientific 1212-1610/1212-1200). Some people prefer to separate the tubes and handle them individually, to minimize the chance of cross-contamination. 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).
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 (i.e., those that grew on tiny ignite slants).
4. Inoculate a piece of mycelium of 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 tester *fl* 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 DNA prep

1. Select 1 strain from each mating type and inoculate a piece of mycelium from the slant culture into 3 ml of liquid VM containing hygromycin (200 µg/ml). 16x125 mm glass culture tubes work well.
2. Incubate at 30˚C with shaking for 1-2 days.
3. Collect fungal tissue using a 12-well vacuum manifold (Millipore), rinse with

sterile water, squeeze dry on filter paper.

4. [The tissue can now be placed in individual 2 ml microfuge tubes, frozen, ground with a glass rod and processed with the Gentra PureGene kit (D-7000A) using their recommendations for fungal tissue.]
5. For high-throughput, 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 (Riplate made by Ritter; obtain from E&K Scientific; #661000). Add a 5 mm stainless steel bead (Qiagen #69989) to each well for grinding.
6. 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 30Hz for 2 x 1 min. Between the two steps, switch the orientation of the plate/tubes as recommended.

Genomic DNA extraction

We are currently using 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 follow their recommended protocol except: before adding RLT buffer 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 (don't allow tissue to thaw!), recap (perhaps with fresh caps or mat), shake and vortex to lyse and suspend tissue thoroughly, and spin at 4,000 rpm for 10 min. For the final elution step, preheat Buffer AE to 60-65˚C and use 50 µl.

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 homokaryon by hybridization with a probe made from the entire deletion cassette. 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.

2. We make probes with the Roche PCR DIG kit, using the cassette DNA as template and a mixture of 4 primers for the flank portions of the cassette. For hybridization, we then add an aliquot of PCR DIG probe (made separately) for the *hph* portion of the cassette. Roche's DIG reagents provide a complete non-radioactive system for probe synthesis and detection. We are not including the entire protocol for this here, assuming that most labs are still using radioactive probes. However, we follow Roche's protocols without significant modifications and will be happy to provide detailed protocols and guidelines upon request.
3. If you make your own KO strains, you can make probes from your cassette DNA and, if desired, from genomic DNA by your favorite methods.
4. For verification of our KO strains in your lab, here are some suggestions (you will need to use the Southern design program for some of the necessary information):
    a. design primers within the *hph* cassette and distal to the flanks used in the cassette and use PCR to see products diagnostic of a particular KO.
    b. PCR the *hph* cassette from pCSN44 and use it to make a probe to verify that you have the correct KO strain (note that only a subset of fragments will be visualized in some cases, depending on the enzyme chosen)
    c. look for the absence of wild-type DNA by either probing the Southern with sequence corresponding to the deleted ORF or by PCR

Media and Solutions

1. **VM agar**

   per100 ml:

50x Vogel's	2ml
1.5% sucrose	1.5g
1% Agar	1g
H_2O	98ml

   VM agar for ignite

   per 100 ml:

50x Vogel's without NH_4NO_3	2ml
1.5% sucrose	1.5g
0.5% L-Proline	0.5g
1% Agar	1g
H_2O	98ml

   Autoclave 20 min (or longer for larger volumes)
   Cool to 60˚C and add selection reagent (hygromycin, ignite, etc.)

2. **50x Vogel's**

   per 2L

$Na_3Citrate.2H_2O$	253.6g
KH_2PO_4	500g
NH_4NO_3	200g (no NH_4NO_3 in 50x Vogel's for ignite media)
$MgSO_4.7H_2O$	20g
$CaCl_2.2H_2O$	10g (dissolve in water first then add later)
Biotin solution	10ml
Trace element	10ml

   Add 10ml chloroform to each bottle

3. **FGS plate + hyg, 2% yeast extract, 100 μg/ml His for plating transformations**

   per liter:

50x Vogel's	20ml
yeast extract	20g
Agar	10g
H_2O	880ml

   autoclave 20 min
   add 100 ml 10x FGS, 2ml 500x L-histidine, and hygromycin (to **300 μg/ml** !!!)

4. **10x FGS additive**
   per 1 liter:

sorbose	200g
fructose	5g
glucose	5g

   filter-sterilize

5. **Regeneration agar with 2% yeast extract, 100 μg/ml His**
   per 600 ml:

50x Vogel's	12ml
sorbitol	109.2g
yeast extract	12g
Agar	6g
H_2O	504ml

   autoclave 20 min and then add 60 ml of 10x FGS additive and 1.2ml of 500X L-histidine

6. **Recovery solution with 2% yeast extract, 100 μg/ml His**
   per 600 ml:

50x vogel's	12ml
yeast extract	12g
H_2O to 600 ml	

   autoclave 20 min and then add 1.2 ml of 500X L-histidine

7. **1x SCM agar (Westergaard's)**
   per liter:

KNO_3	1.0g
K_2HPO_4	0.7g
KH_2PO_4	0.5g

$MgSO_4.7H_2O$	0.5g
$CaCl_2$	0.1g
NaCl	0.1g
Biotin (50ug/ml)	0.1ml
Trace elements	0.1ml
Sucrose	15g
Agar	10g
H_2O	

Autoclave 20min and add selection additive after cooling down to 60˚C

8. **Biotin solution**

5mg Biotin in 100ml 50% (v/v) EtOH
Filter sterilize and refrigerate

9. **Trace Elements**

per 100 ml:

Citric acid.H_2O	5g
$ZnSO_4.7H_2O$	5g
$Fe(NH_4)_2(SO_4).6H_2O$	1g
$CuSO_4.5H_2O$	0.25g
$MnSO_4.H_2O$	0.05g
H_3BO_3	0.05g
$NaMoO_4.2H_2O$	0.05g
H_2O to 100 ml	

Filter sterilize and refrigerate