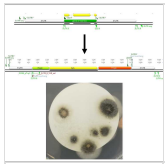

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Fungal gene knockout with Agrobacterium tumefaciens using Gibson assembly

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

This method uses Agrobacterium tumefaciens to transfer a piece of TDNA containing a resistance gene (hph for hygromycin in this particular case) flanked by homologous regions of the gene you want to knock out into the fungal spore.

The process is broken down into following steps:

- Assembly of the knockout construct via Gibson assembly
- Transformation into E.coli
- Plasmid harvest and transformation into Agrobacterium tumefaciens
- TDNA transfer into fungal spores
- Verifying knockout

EXTERNAL LINK

https://www.mdpi.com/2309-608X/6/4/314

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PROTOCOL CITATION

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KEYWORDS

Agrobacterium, gene replacement, knockout, fungi

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20207

MATERIALS TEXT

To make induction media you need to prepare several solutions:

A	B
Name	component
1.25 M KH2PO4	170.1 g of KH2PO4 in 1 litre of H2O --> autoclave
1.25 M K2HPO4	217.7 g of K2HPO4 in 1 litre of H2O --> autoclave
K-buffer	Add 1.25 M KH2PO4 to 1.25 M K2HPO4 until pH 4.8 is reached
MN buffer	30 g of MgSO4 x 7H2O and 15 g of NaCl in 1 litre of H2O --> autoclave
Trace elements for IM medium	100 mg of ZnSO4 x 7H2O, 100 mg of CuSO4 x 5H2O, 100 mg of H3BO3, 100 mg of MnSO4 x H2O, 100 mg of Na2MoO4 x 2H2O in 1 litre of water --> autoclave
1 M MES (mw 213.25 g/mol)	213.25 g of MES in 1 litre of H2O. Adjust pH to 5.5. Filter sterilize, can be stored in the dark for 1 month or freeze in aliquots. Make 40 ml aliquots
1% CaCl2 (wt/vol)	10 g of CaCl2 x 2H2O in 1 litre H2O --> autoclave
0.01% FeSO4 (wt/vol)	0.1 g FeSO4 x 7H2O in 1 litre H2O --> filter sterilize
20% glucose (wt/vol)	200 g of glucose in 1 litre H2O --> autoclave
20% NH4NO3 (wt/vol)	200 g of NH4NO3 in 1 litre H2O --> autoclave
0.2 M Acetosyringone	396 mg AS in 10 ml DMSO --> filter sterilize and aliquot, try to minimize freeze thaw
IM (liquid)	- 0.8 ml K-buffer - 20 ml MN buffer - 1 ml of 1% (wt/vol) CaCl2 x 2H2O - 10 ml of 0.01% (wt/vol) FeSO4 - 5 ml of trace elements for IM medium - 2.5 ml of 20% (wt/vol) NH4NO3 - 10 ml of 50% (vol/vol) glycerol - 40 ml of 1 M MES, pH 5.5 - 10 ml of 20% (wt/vol) glucose - fill to 1 litre with H2O
IM (solid)	- 0.8 ml K-buffer - 20 ml MN buffer - 1 ml of 1% (wt/vol) CaCl2 x 2H2O - 10 ml of 0.01% (wt/vol) FeSO4 - 5 ml of trace elements for IM medium - 2.5 ml of 20% (wt/vol) NH4NO3 - 10 ml of 50% (vol/vol) glycerol - 40 ml of 1 M MES, pH 5.5 - 5 ml of 20% (wt/vol) glucose - 15 g Agar - fill to 1 litre with H2O

Solutions needed

PCR of flanking regions

1 Depending on your fungal organism you may have to try different sizes. A good starting length is 1000 bp up- and downstream of your gene of interest.

Use a proof reading polymerase!

The primers we use to amplify the 5'- and 3' UTRs will carry tails which overlap with our plasmid backbone and the selection marker cassette.

Design your primers as 15-20 bp matching the region of interest which we will use as the homologous fragments (the 1000 bp up- and downstream of the sequence we want to replace) and add the following sequences as 5'-extensions to your primers:

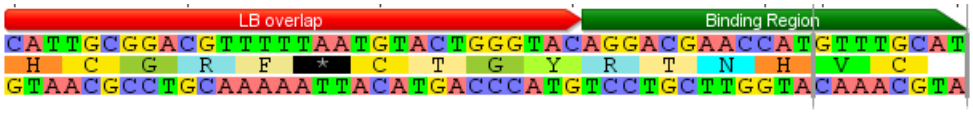
5'UTR Forward primer extension: CATTGCGGACGTTTTTAATGTACTGGGTACC (overlaps with the Left Border of the plasmid backbone)
5'UTR Reverse primer extension: AGTGCTCCTTCAATATCATCTTCTGGGTAC (overlaps with the PtrpC promoter of the resistance cassette)
3'UTR Forward primer extension: AATGCACAGGTACACTTGTTTAGAGGGTAC (overlaps with the TtrpC terminator of the resistance cassette)
3'UTR Reverse primer extension: ACCCAAATCAAGTTTTTTGGGGTCGGGTACC (overlaps with the Right Border of the plasmid backbone)

Use your polymerase manufacturers manual for PCR conditions.
10-20 ul assays should give you enough fragment for cloning.

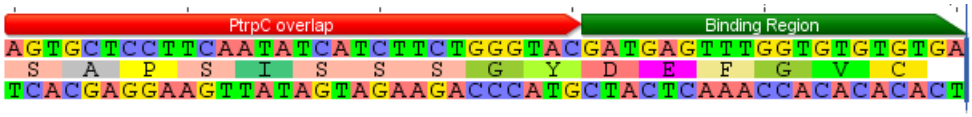
Example primers:

5' UTR Forward: **CATTGCGGACGTTTTTAATGTACTGGGTAC***AGGACGAACCATGTTTGCAT*
5' UTR Reverse: **AGTGCTCCTTCAATATCATCTTCTGGGTAC***GATGAGTTTGGTGTGTGTGA*

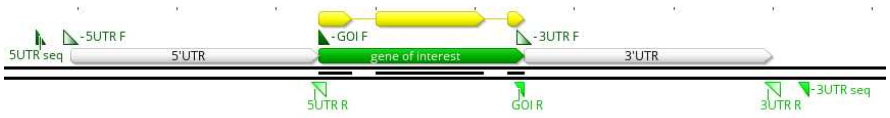
Bold: 5' primer extension
Italics: gene (UTR) specific sequence where primer binds



Full 5UTR F primer example



Full 5UTR R primer example



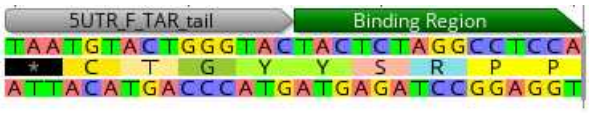
Gene of interest in genomic context

1.1 OPTIONAL (2 step PCR to save money on primers)

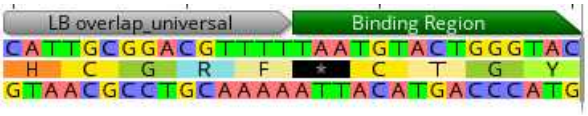
If you are going to do this on a regular basis, ordering long primers like those above becomes expensive quickly. We therefore split the PCR process into two rounds. The first uses a gene specific primer with a shorter tail. That PCR product then becomes the template for a second PCR with universal primers which bind to the short tail introduced during PCR 1 and carry their own tail to complete the full overlap sequence.

Example:

5'UTR forward primer (gene specific) for PCR1:



5'UTR universal primer for PCR2:



Full overlap after PCR2:

As above, you design your respective primers with 15-20 bp gene specific sequence and then add the following as 5' extensions:

5'UTR forward primer extension: TAATGTACTGGGTAC

5'UTR reverse primer extension: TCATCTTCTGGGTAC

3'UTR forward primer extension: TTGTTTAGAGGGTAC

3'UTR reverse primer extension: TTTGGGGTCGGGTAC

Universal 5'UTR forward primer: **CATTGCGGACGTTTTT** *AATGTACTGGGTAC*

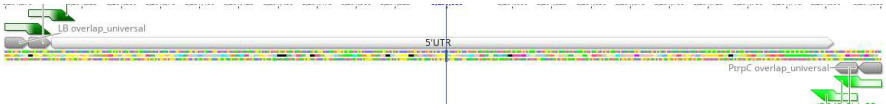
Universal 5'UTR reverse primer: **AGTGCTCCTTCAATA** *TCATCTTCTGGGTAC*

Universal 3'UTR forward primer: **AATGCACAGGTACAC** *TTGTTTAGAGGGTAC*

Universal 3'UTR reverse primer: **ACCCAAATCAAGTTT** *TTTGGGGTCGGGTAC*

Bold: 5' primer extension
Italics: binds to 5'extension of first round primer

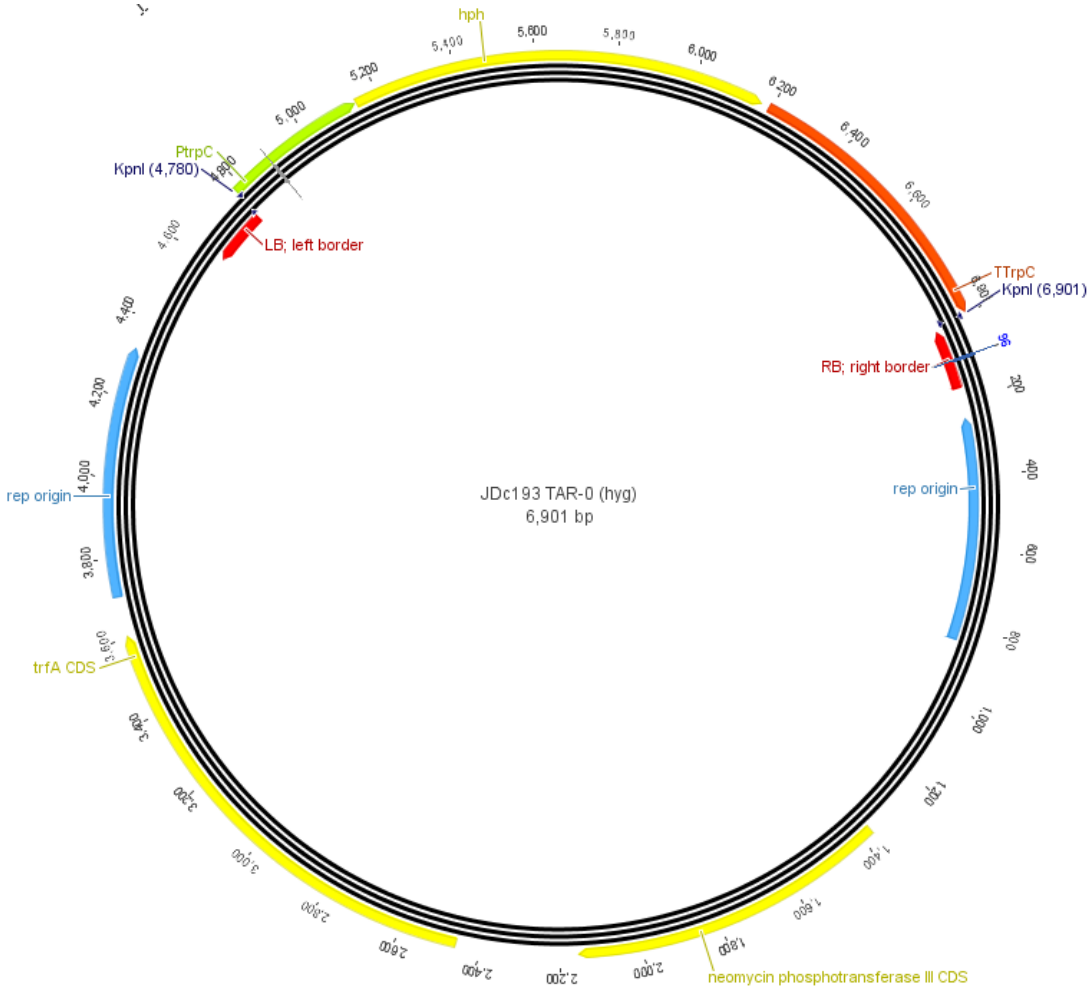
Example 5'UTR after PCR 2 with full overlaps:



Digestion of Backbone with KpnI

2 We need to open the plasmid up to separate the backbone from the resistance cassette (PtrpC-hph-TtrpC). The plasmid was designed to contain two KpnI restriction sites flanking the resistance cassette in order to accomplish this.
Follow the restriction enzyme manufacturers recommendations.
Digest about 1ug of plasmid.

Plasmid in genbank and geneious format:
pTAR-0-hyg.gb pTAR-0-hyg.geneious



Cleanup of flanking regions, plasmid backbone and resistance cassette

3 After PCR of flanking regions and plasmid digestion run all reactions on a gel.
We do this for two reasons:

1. To check if the PCR of the flanking regions actually worked
2. To separate the digested plasmid fragments from any undigested plasmid (which would otherwise lead to false positive colonies later on which do not contain the assembled flanking regions).

Use your preferred gel extraction kit for this and elute fragments in as small a volume as you can get away with.

3.1 OPTIONAL (but improves efficiency) Cleanup of gel extracted fragment

I have had mixed results using the gel extracted fragments directly with Gibson assembly. The Nanodrop results are usually not that great. I therefore now use the Zyppy Clean and Concentrator kit to clean up the gel extracted fragment. This also allows you to elute in as little as 5 ul efficiently.

Gibson assembly of fragments into backbone

4 We use Gibson assembly to create our knockout construct. As shown in the picture below, we want a construct that looks as follows:

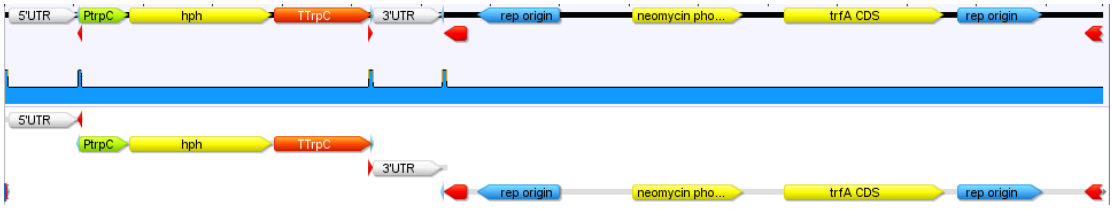
plasmid backbone - LB - 5'UTR - resistance cassette - 3'UTR - RB - plasmid backbone.

Follow your Gibson enzyme mix manufacturer's instructions.

In the case of NEB's **NEBuilder® HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit** use the NEBioCalculator and following table as a guide. The manual suggests to use 50 fmol per fragment for the assembly of 4 or more fragments.

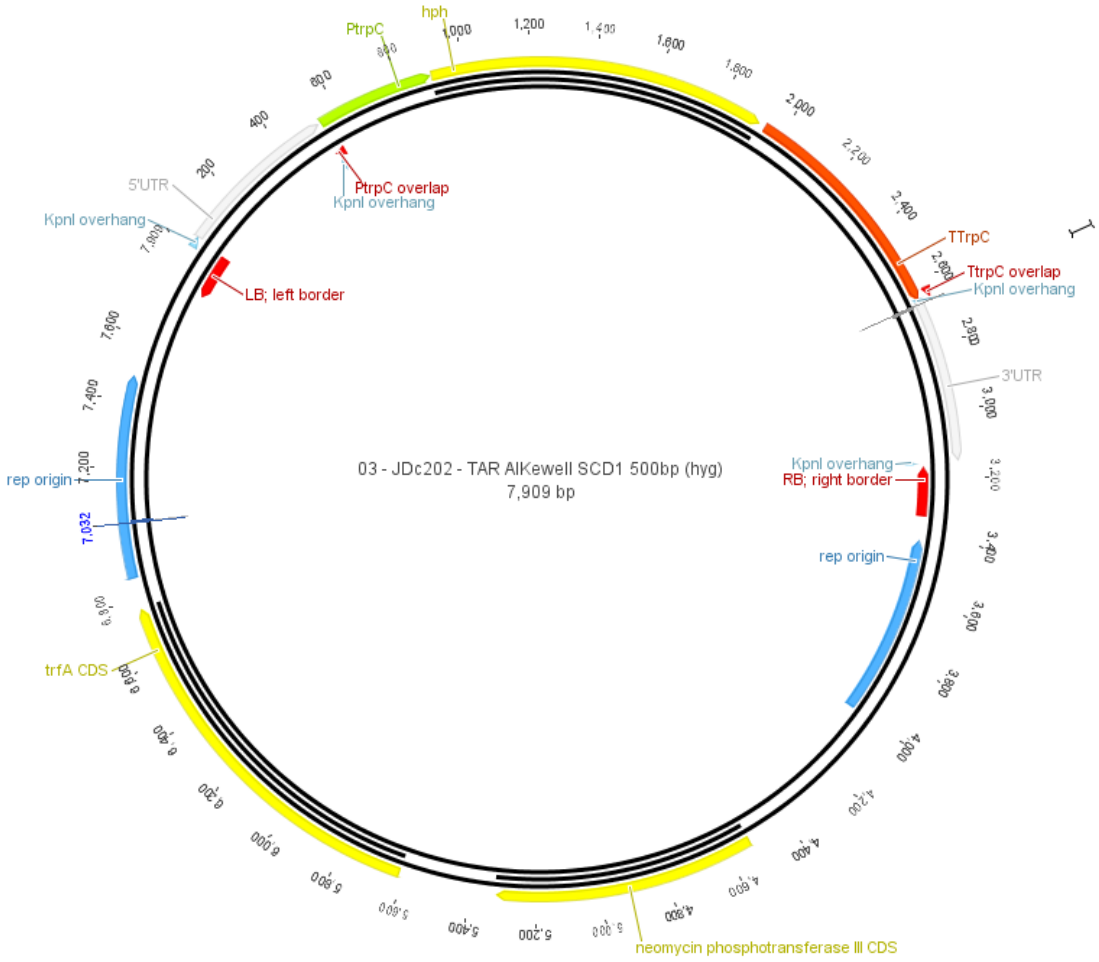
A	B	C	D
2x NEBuilder mix	5		
hph cassette (31 ng/ul)	0.7	22 ng	50 fmol
backbone (125 ng/ul)	0.5	75 ng	50 fmol
5'UTR 500 bp (25 ng/ul)	0.4	10 ng	50 fmol
3'UTR 500 bp (24 ng/ul)	0.4	10 ng	50 fmol
H2O	3		

1 hour at 50°C followed by putting the fragments on ice until transformation into E.coli.



Schematic of the expected assembly.

We should end up with a plasmid like this:



Gibson assembled KO construct

Transformation of assembly mix into E.coli

5 Transform 1 - 5 ul of the Gibson assembly mix into 50 ul thermocompetent E.coli

- Combine **1 - 5 ul NEBuilder mix** with **50 ul thermocompetent E.coli**
- Incubate **on ice for 10-30 minutes**
- heat shock for **30-40 seconds at 42°C** in a waterbath
- Incubate **on ice for 5-10 mintues**
- add **750 ul SOC** medium
- Incubate for **1 hour** on a shaker incubator at **37°C** and **250 rpm**

Plate transformants

6
- Concentrate cells by centrifugation at **2000 x g for 2-5 minutes**
- discard most of the supernatant
- resuspend cell pellet in **100-200 ul** leftover supernatant
- plate cell mixture on LB plates containing **30 ug/ml kanamycin** (try different amounts to avoid ending up with too many colonies, for example: one plate with 5 ul, one with 20 ul and one with the rest)
- incubate **overnight at 37°C**

Depending on the competency of your E.coli cells, you may be able to use them directly without spinning and concentrating them first.

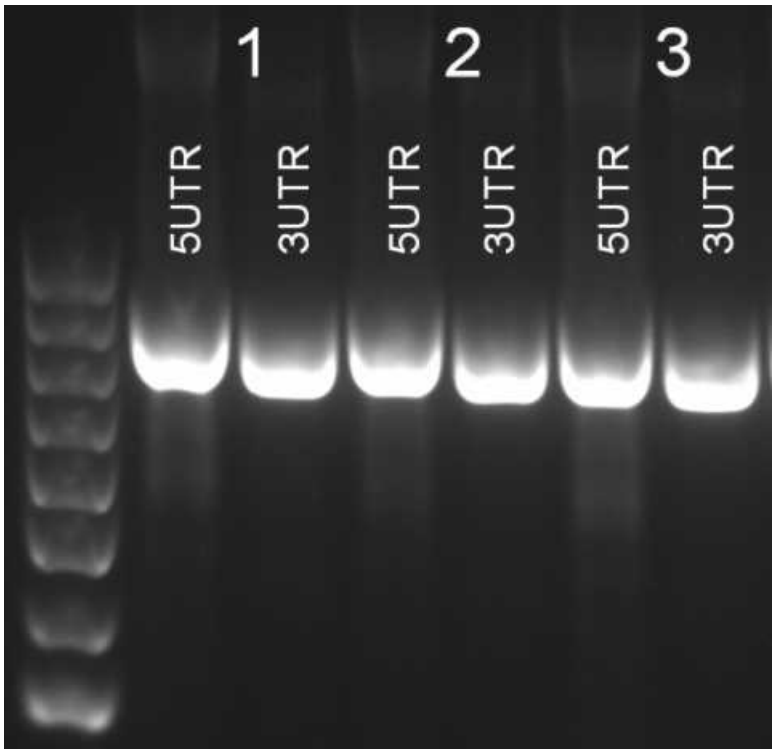
Colony PCR to screen for correct transformants

7 Growing colonies after overnight incubation point to transformants carrying the resistance gene. But in order to make sure those are not just contamination from carried over plasmid backbone we will run two PCR reactions on each

colony (multiplex works as well, but is better for shorter flanking regions) to check for presence of assembled flanking regions.

A	B	C
ID	sequence	binds at
JD281	GGCAGTGACGTCATCG	plasmid backbone (RB)
JD333	CCGTAACACCCAATACGC	TtrpC
JD306	*AGACTGAGGAATCCGCTC*	*TtrpC (optional primer for multiplex)*
JD288	GGCTGATCTGACCAGTTG	PtrpC
JD445	GCCTTACAACGGCTCTC	plasmid backbone (LB)

For a 500 bp flanking region we expect following bands:
JD281 + JD333 = 828 bp
(JD281 + JD306 = 1124 bp - use this pair if you want to multiplex all 4 primers to get fragments of more different sizes - JD281, JD306, JD288, JD445)
JD288 + JD445 = 848bp



Gel of flanking regions (not multiplexed), two lanes per clone.

Harvest plasmid and sequence

8 If colony PCR gives you bands of the expected size grow the respective colonies in **5 ml LB** medium containing kanamycin at a final concentration of 30 ug/ml overnight at **37°C at 250 rpm**.

The next morning combine **700 ul overnight culture with 300 ul 50% glycerol** and store as backup in the -80°C freezer.

Perform a mini prep on the other 4 ml with your favourite kit or method.
Adjust plasmid concentration after prep to about 100 ng/ul.

Sequence your assembled plasmid across the flanking regions using following primers:

JD445	GCCTTACAACGGCTCTC	from backbone into 5' flanking region
JD288	GGCTGATCTGACCAGTTG	from PtrpC into 5' flanking region
JD333	CCGTAACACCCAATACGC	from TtrpC into 3' flanking region
JD281	GGCAGTGACGTCATCG	from backbone into 3' flanking region

Electroporate plasmid into Agrobacterium

9 Electroporate your sequence verified plasmid into your *Agrobacterium tumefaciens* strain of choice.

Electroporation of Agrobacterium tumefaciens

You may want to run another colony PCR on Agrobacterium colonies to verify that the colony contains your knockout construct.

10 Verify your Agro colonies by PCR using the same primers as in step 7 above.

Fungal transformation

11
- Grow transformed Agrobacterium (strain AGL1) on a plate (**LB + rifampicin 50 ug/ml + kanamycin 30 ug/ml**) at **25°C** until colonies appear.
- Then inoculate a colony from that plate into **50 ml LB + rifampicin 50 ug/ml + kanamycin 30 ug/ml** and grow at **27°C at 250 rpm overnight**.

12
- The next morning check if the inoculated media is turbid and if so, transfer to a 50 ml falcon tube and **spin at 3000 x g at 4°C for 20-30 minutes.**
- Resuspend pellet in **50 ml Induction Medium (IM)**
- Measure OD600
- **spin at 3000 x g at 4°C for 20-30 minutes.**
- Resuspend pellet in **IM so the final OD600 is ~0.5** (if it was 0.3 at first measurement, resuspend in 30 ml IM, if it was 0.4, resuspend in 40 ml etc.)
- Add **0.2 M Acetosyringone to a final concentration of 0.2 mM** (for 50 ml add 50 ul, etc.)
- Incubate at **150 rpm at 27°C for about 5 hours**

13 Harvest fungal spores the way you usually harvest them. In our case, we flush a plate containing spore producing fungus with sterile H2O and pass the water through a syringe stuffed with sterile cotton wool. The filtrate then gets spun at 3000 - 5000 x g for 20 minutes at 4°C. The spore pellet, depending on how big it is, get resuspended in sterile H2O and spores are counted using a haemocytometer.

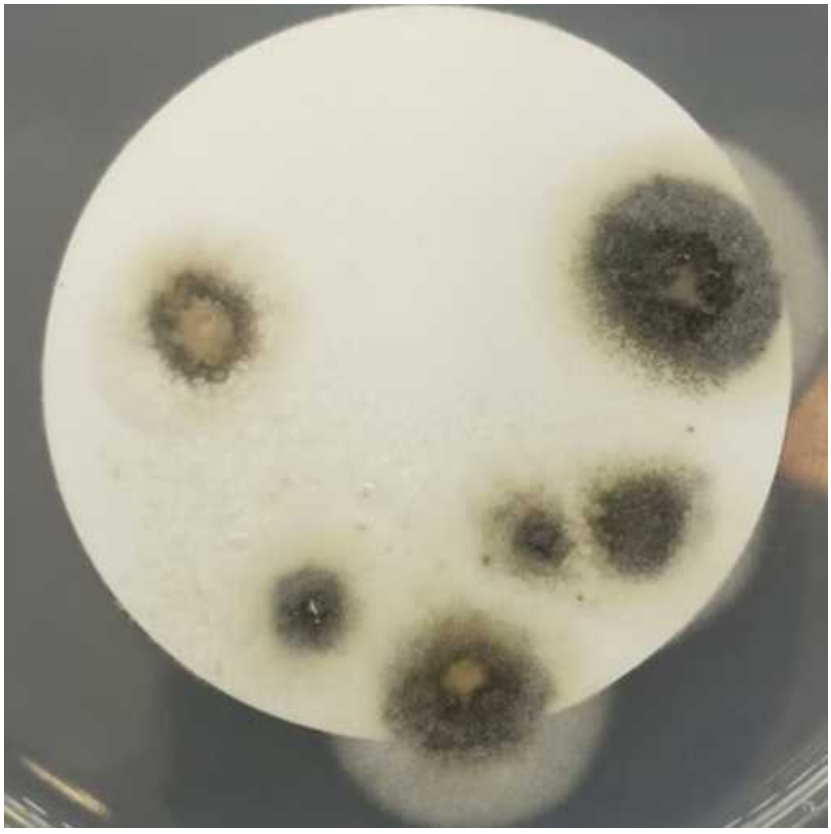
14
- mix **100 ul spores** (you'll have to find out what concentration works best for you) with **100 ul Agrobacterium** solution (**OD600 ~0.5**).
- Put a **nitrocellulose membrane** (Whatman cellulose nitrate membrane, 0.45 um, 47 mm diameter) onto an I**M Agar plate containing 0.2 mM Acetosyringone**.
- **Spread 200 ul Spore / Agrobacterium** solution onto membrane (Use 3 - 10 plates per transformation)
- Wrap plate with parafilm or clingwrap and store at **25°C in the dark for 2 - 5 days**
- Transfer membrane to **1/2 PDA hyg 50 ug/ml cef 50 ug/ml strep 30 ug/ml plates**, wrap and put back at 25°C in the dark
- Observe plates and wait for colonies to emerge

A	B	C
Fungus	spore concentration	marker
A. rabiei me14	2 x 10^6 spores/ml	Hygromycin
A. lentis Kewell	2 x 10^7 spores/ml	Hygromycin

Spore concentration for transformation

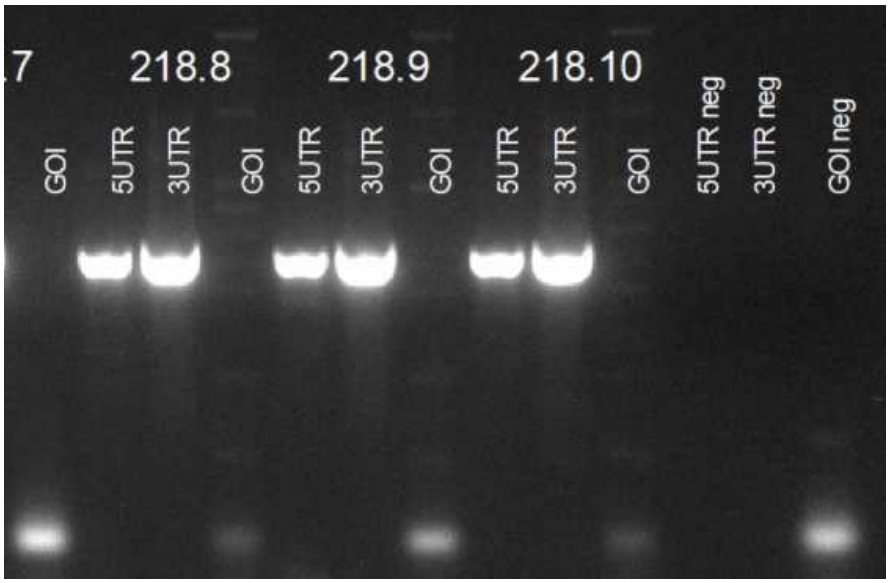
Verify transformants

15 Once Colonies appear on your selective plates you need to transfer them to fresh selective plates. One plate per colony. Let them grow for a while and then do a Quick DNA extraction followed by PCR across your flanking regions (5UTR seq + JD288, 3UTR seq + JD333) as well your gene of interest (which should now be replaced).



Colonies of transformed fungus after membrane transfer to selective plate

Gene of interest replaced with Hygromycin cassette.



PCR to check for correct insertion (5UTR: genome into marker cassette, 3UTR: genome into marker cassette, GOI: gene of interest). You want bands for both UTRs and no band for GOI, which means the marker cassette has been inserted at the location of the gene of interest –> correct KO.

16 OPTIONAL (but suggested) - sequence KO

Amplify a region starting outside the UTR sequences used to create your KO clone and sequence from there into the marker cassette (Primers 5UTR seq + JD288 and 3UTR seq + JD333 as denoted in the diagram above.