Genetic transformation of filamentous fungi v1.0

Duration: 1 h at day 1 + 6-8 h time span at 2. day

Needed items

Fungus 3-point inoculated on plates – host strain
Drigalski spatula
70 % and 96-99.9 % EtOH
Glass beakers
Optional: counting-chamber
Solutions (see text for details): MM, APB, ATB, PCT, Milli-Q H_2O, TM
Paper towel
Incubator with and without shaking
Spoon (tea size)
Sterile Mira-cloth filters (funnel+miracloth+alu foil)
Glucanex or other cell-wall degrading enzymes
Magnetic stirrer
Magnets
50 ml sterile falcon tubes
0.20 and 0.45 µm filters
20 ml syringes
Microscope
Safety Cabinet/LAF bench
Large Centrifuge + Rotor for falcon tubes
Microcentrifuge (capable of 14.000x g)
Rack for microcentrifuge tubes
sterile 1.5 ml microcentrifuge tubes
sterile 15 ml tubes
5 ml pipette and sterile tips
1 ml pipette and sterile tips
20-200 µl pipette and sterile tips
2-20 µl pipette and sterile tips
Sterile tooth picks/inoculation needle – post transformation
Minimal medium agar plates – post transformation

1. **Initiation:** the host strain for transformation is grown as three-point stabs on mineral media plates (MM) supplemented with growth requirements. MM will for convenience throughout the protocol refer to mineral medium with any supplements included. If the strain displays poor sporulation, increase the number of plates accordingly.

2. All solutions listed in **Buffers and solutions** should be sterile and ready for use in step 3. If step 5B is followed, sterile Mira cloth in funnels packed in alu foil should be ready for use.

3. The experiment and handling of open cultures should be carried out in a Laminar Airflow Bench/Safety cabinet to protect you and the experiment.

1. Day

4. **Inoculation:** Collect the conidia from plate(s) having the three colonies – strains with poor sporulation require more plates, whereas one plate with wild type is sufficient (4-5 days growth). Prepare a sterile 500 ml shake flask (preferably without baffles) containing 100 ml MM (or YPD or CYA for fungal species which are not thriving in MM – remember to supplement complex/rich media with growth supplements). Add 5 ml of the MM to the dish with fungus and firmly, but not too violently, rub off conidia from the colonies with a sterile Drigalsky spatula. The conidial suspension, in the end giving around 10^8 spores/100 ml is pipetted to the shake flask. In the case of counting the conidia is required, remember to withdraw 10 µl sample and dilute accordingly to estimate the spore-count in a counting chamber. Too few spores in the culture result in pelleted growth.

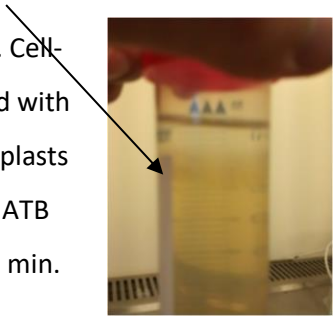
5. The cultures are incubated at 25 or 30 or 37 °C (depending on the species) with 150 rpm of shaking overnight (14-40 hours) giving a minimum of 2x 2 g mycelia. Usually, biomass is filling the cone of the falcon tube (until 5 ml), but it is recommended to try different amounts until the optimal conditions has been reached for your fungus.

2. Day

6. **Mycelial harvest**: There are two procedures for harvesting the fungal biomass. **A:** Retain biomass by **filtering** in sterile Mira cloth (recommended). **B**: **Centrifugation** at 3300x g for 5 min in sterile 50 ml falcon tubes. Remember to balance tubes within a max discrepancy of 0.1 g. Discard the supernatant by gentle decanting – this step is repeated.

7. **Wash the mycelia** with ultra-pure sterile water (and optional some ***Aspergillus* protoplastation buffer (APB))** to remove residual glucose from the mycelia, which can inhibit protoplastation. In the case of filtered biomass, the washed mycelium is transferred to new Falcon tubes with a sterile spoon. Be careful not to touch the interior of the tubes with the non-sterile spoon handle.

8. **Protoplastation**: Resuspend the mycelium, at least 1-2 g (lower than the 5 ml mark on the Falcon tube, in 10 ml filter-sterilized (0.45 µm filters) APB containing 40 mg Glucanex/ml (or create Enzyme mixture with Beta-glucanase). The Glucanex is dissolved in APB with very **gentle** magnetic stirring.
9. Shake/incubate enzyme-mycelium mix at 30°C and 150 rpm for approximately 2-3 hours. For proper mixing keep a 0 to 15° angle on the Falcon tube. Take 5 µL samples regularly (every 30-60 min) to check the cell-wall digestion and the release of protoplasts in the microscope. If transformation with antibiotic selection is used, please see **26.** for top-agar preparation.
10. The number and quality of protoplasts are evaluated in the microscope, since a too diluted batch may not produce enough transformants. Approved protoplast solutions are diluted by pouring APB up to 40 ml. Tubes are balanced and an overlay of max. 5 mL *Aspergillus* transformation buffer (ATB) diluted to ½x with sterile Milli-Q H_2O (i.e. use a 5 mL pipette) is carefully placed on top of the APB. Balance before making the overlay. Avoid using highest in acceleration and brake settings since it may disturb the layer.
11. In the interphase of the two liquids, a halo of white slurry consisting of concentrated protoplasts should be observed. Cell-wall debris is collected in the pellet, and some can be mixed with the protoplasts, which can be ignored. Withdraw the protoplasts with the pipette and place them in a new Falcon tube. Add ATB up to 40 ml, and pellet the protoplasts at 3000x g for 10-15 min. Discard the supernatant by decanting.



12. *Optional wash:* Wash the protoplasts gently in ATB. This removes impurities such as enzymes, e.g. with nuclease activity that can decrease the efficiency of the following genetic transformation. However, this results in loss of protoplasts.
13. Resuspend the protoplasts in ~1 ml ATB, or less to obtain a more concentrated protoplast solution. Use pipette tip with large nozzle to avoid shearing and stressing the protoplasts. Aliquot protoplasts in sterile microcentrifuge tubes for storage at -80 °C or keep cold at 4 °C until use, for less than a week. Frozen protoplasts can be thawed at room temperature before use. Move to 20.
14. **Alternative for Protoplastation by Filtration 15.-20.:** (This method has shown to be well-suited for most *Aspergillus* Section Nigri)

15. When most, or all, of the mycelia is digested, filter the culture through sterile Miracloth in sterile falcon tubes.
16. Spin the filtrate at 800x g for 10 minutes to pellet the cells, decant supernatant immediately after centrifugation, even at centrifuge, since the pellet is quite fragile and dissolves easily.
17. Add 25 ml of ST solution (1.0 M sorbitol; 50 mM Tris, pH 8.0) or ATB, resuspend the cells well and spin 800x g for 10 minutes.
18. *Optional wash:* Add 25 ml of ST solution (1.0 M sorbitol; 50 mM Tris, pH 8.0) or ATB, resuspend the cells well and spin 800x g for 10 minutes.
19. Resuspend the cells in 10 ml of STC (1.0 M sorbitol; 50 mM Tris, pH 8.0; 50 mM $CaCl_2$) and centrifugate. Pellet may be even more fragile/loose. Remove the STC wash and resuspend the pellet in ~1 ml of STC. Use pipette tip with large nozzle to avoid shearing and stressing the protoplasts. Aliquot protoplasts in sterile microcentrifuge tubes for storage at -80 °C or keep cold at 4 °C until use, for less than a week. Frozen protoplasts can be thawed at room temperature before use.
20. *Optional:* Count the protoplasts and add STC to level concentration to $1x10^7$/ml. The appearance should be milky.
21. **Genetic transformation - Simple with nutritional marker:** Considerations; Aliquots of 50- 200 µl protoplasts (~10^5-10^7/ml) are typically used. The volume of DNA in water should preferably be kept below 25 % of the total volume of DNA-protoplast mix to avoid osmotic stress in protoplasts. Then add more ATB not to disrupt the protoplasts. The amount of DNA needed for a successful transformation varies with the type of the DNA substrate.
    a. Add 1.5-5 µg of DNA per substrate (i.e. bipartite 2x (1-5)µg). If CRISPR/Cas is used, lower concentrations may be used.
    b. For AMA1-based plasmids: add 100-1000 ng DNA.
    c. Remember to include a positive (AMA1 vector with marker) and negative control plate (no DNA)
22. Gently mix protoplasts and DNA in a microcentrifuge tube according to **20**. And add 150 µL PCT with large-nozzle pipette tip and stir plus flip with finger on tube. Do not mix in the tip! Or reaction-volume loss will happen.
23. Incubate reaction at room temperature for at least 10 min.

24. Add 250 µL ATB and transfer the transformation mix to selective transformation medium (TM) labelled with appropriate identifier on the side of the petri dish. Distribute it lightly on the plate and leave to dry for 30 min.
25. Put plates in microperforated bags, bottom down - lid up, at preferred temperature, which depends on the host and the expected sensitivity of the mutants (e.g. temperature sensitivity). Check for growth every day. Do not turn plates around. 3-8 days for engineered mutant colonies to show depending on the temperature, and 2-3 days for positive control colonies to appear. Check for transformants and count. Describe any phenotypes and make records of data. Move to **30.**

26. **Transformation – Antibiotics:** Aliquot 1 ml PCT to sterile 15 ml tubes while incubating – one per reaction. Add the DNA and protoplast suspension to the PCT. Shake gently. Incubate for 5-10 minutes at room temperature. Dilute each reaction with 3 ml ATB. Prior to this experiment and selection, we recommend that you perform a spot assay for your fungus for varying concentrations of the antibiotic to detect the sensitivity level for the drug.
27. During the protoplastation phase or before thawing protoplasts, **prepare the agar transformation medium** (TM, sucrose based for osmotic stability in protoplasts - p.7). When the medium has cooled to 80-60 °C add any supplements (0.22 µm filters) and antibiotic. The latter should be added to a low quantity vessel to minimize exposure of drug and avoid reheating and reuse of media slants. If you want antibiotics in the TM plate, make the number plates needed for transformations) by pouring ~25 ml agar in each Petri dish. Label with appropriate identifier on the side of the petri dish. Ensure that enough agar for the number of transformations x 12 ml is saved for later use as top agar in step **27**. This remaining TM is kept molten at 45-60 °C until use, max 1 day.
28. Fill the reaction tube with the molten agar media, amounting to approximately 12 ml, secure the lid, and mix rapidly by inverting the tube twice. This is the top agar. Pour directly the top agar on pre-made TM plates.
    NOTE: the agar medium should probably have a temperature of around 40-45 °C. If it is too warm, the cells will die, and if it is too cold, the top agar will solidify in the tube and create a mess on the transformation plates.

29. Let the plates settle for a few minutes. Put plates in microperforated bags, bottom down - lid up, at preferred temperature, which depends on the host and the expected sensitivity of the mutants (e.g. temperature sensitivity). Check for growth every day. Do not turn plates around. 3-8 days for engineered mutant colonies to show depending on the temperature, and 2-3 days for positive control colonies to appear. Check for transformants and count. Describe any phenotypes and make records of data.

30. **Re-streaking:** Have MM plates with appropriate supplements ready for re-streaking of transformants. If different phenotypes are observed, pick different transformans and minimum four for clean-streak and analysis (for NHEJ-mutant host or CRISPR/Cas based), more for wild-type host/random integrations. Take sterile toothpick or inoculation needle and gently inoculate the transformant hereby picking conidia, a few as possible to reduce the risk of transferring two different types of nuclei, either from diploid strain or a heterokaryotic mycelium. Make a zigzag line with the toothpick on the fresh agar plate which will facilitate obtaining single-spore based colonies that likely will be homokaryons. Alternatively used a dissection microscope to pick single spores.

31. When you believe they are single-spore colonies, inoculate as three-point inoculations and incubate at optimum temperature before doing colony PCR or Purification of fungal gDNA. Wen they have been verified, e.g by PCR make another plate for "Preserving your filamentous fungus". It is recommended to save at least three independent transformants as stocks if possible.

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Buffers and solutions:

All solutions are diluted with Milli-Q H_2O, and Autoclavation is done at 121 °C for 20 min.

Trace element solution (1 l stock solution)

Add: *0.4 g* $CuSO_4 \cdot 5H_2O$; *0.04 g* $Na_2B_4O_7 \cdot 10H_2O$; *0.8 g* $FeSO_4 \cdot 7H_2O$; *0.8 g* $MnSO_4 \cdot 2H_2O$; *0.8 g* $Na_2MoO_4 \cdot 2H_2O$; and *8.0 g* $ZnSO_4 \cdot 7H_2O$.

Mineral Mix (1 l stock solution)

Add: *26 g* KCl; *26 g* $MgSO_4 \cdot 7H_2O$; and *76 g* KH_2PO_4; and *50 ml* Trace element solution

***Aspergillus* protoplastation buffer (APB)** (4x 0.5 l)

Final conc: 1.1 M $MgSO_4$ and 10 mM Na-phosphate buffer. (Hint: use 1 M solutions of Na_2HPO_4 and NaH_2PO_4 to prepare the sodium phosphate buffer). pH is adjusted with 2 N NaOH to 5.8.

For 2 l

First the 10 mM Na-phosphate buffer (pH ~5.8) is made

1M Na_2HPO_4	1.58 mL
1M NaH_2PO_4	18.42 mL
Milli Q water	up to 2000 mL

Then the APB buffer:

$MgSO_4$ (MW=246.48)	542.3g

10 mM Na-phosphate buffer not too close to 2000 ml – since you need to leave room for dissolving the solution by heating and adjustment of pH to 5.8 with 2M NaOH. It requires patience and time.

- We recommend to filter-sterilize into sterile 500 mL bottles (250ml filter cups and vacuum can be used for the 2 l).

***Aspergillus* transformation buffer (ATB)** (4x 0.5 l)

Final conc: 1.2 M Sorbitol; 50 mM $CaCl_2 \cdot 2\ H_2O$; 20 mM Tris; and 0.6 M KCl. pH is adjusted with 2 N HCl to 7.2.

For 2 l

D-Sorbitol	(MW=182.17)	437.2 g
$CaCl_2$	(MW=147.02g/mol)	14.7 g
TRIS	(MW=121.14g/mol)	4.9 g
KCl	(MW=74.56)	89.5 g

Milli Q water up to 2000 ml, with space for pH adjustment with 2 N HCl to 7.2.

- Autoclave in 500mL bottles.

PCT (200 ml stock solution)

Final conc: 50 % w/vol PEG 8000 (4000, 6000 and other PEG can also be used); 50 mM $CaCl_2$; 20 mM Tris; and 0.6 M KCl. pH is adjusted with 2 N HCl to 7.5. Autoclave and Store PCT at 4 °C.

For 0.2l	PEG 8000 or other PEG		100 g
	$CaCl_2$	(MW=147.02g/mol)	1.47 g
	TRIS	(MW=121.14g/mol)	0.49 g
	KCl	(MW=74.56)	8.95 g

20x nitrate salts (1000 ml stock solution). Autoclave solution.

$NaNO_3$	120 g
KCl,	10,4 g
$MgSO_4*7H_2O$	10,4 g
KH_2PO4,	30,4 g
Milli Q,	Up to 1000L

20 %-w/vol D-glucose

Optional, but useful solutions: 1 M Tris-HCl pH 8.0; 1 M Na_2HPO_4; and 1 M NaH_2PO_4

Media:

NOTE: Depending on the strain used, certain supplements are required for growth (see table 1 p. 8). Not all of these compounds are suitable as water based stock solution (e.g. uracil and 5-FOA), and are therefore added in low quantities to 50-100 ml of heated Milli-Q H_2O (~80-100 °C) in a beaker glass, with stirring and covered with alu foil, then filter-sterilize in the autoclaved MM.

Thus, when adding compounds to basic MM remember to make room for the volume of the supplements.

Minimal medium (MM) for submerged cultivation (1 l stock)

The following solutions are mixed in Milli-Q water, leave space for sterile-filtered supplements – e.g. 100 ml to 1 l for dissolving uracil and 5-FOA:

Mineral mix	20 ml
20x Nitrate salts	50 ml
Thiamine 1%	1 ml
20 %-w/vol glucose	50 ml

500 ml shake flasks with or without baffles are autoclaved empty with stoppers and alu foil for 20 minutes at 121 °C. MM is added to the shake flasks before inoculation in a LAF bench, typically 100 ml is used.

Basic solid MM for cultivating strains – glucose based (1 l = 40 plates)

The following is mixed with Milli-Q water to a volume of 900 ml (around 100 ml left for dissolving and filter-sterilizing media supplements such uracil and 5-FOA):

Mineral mix (50x):	20 ml
20x Nitrate salts	50 ml
Thiamine 1%	1 ml
20 %-w/vol glucose:	50 ml
Agar	20 g

Autoclaved for 20 minutes at 121 °C.

Add media supplements to the Milli-Q H_2O and filter-sterilize.

Basic solid TM for genetic transformation – sucrose based (0.5 l)

The following is mixed and added Milli-Q water to a final volume of 450 ml (50 ml left for filer-sterilized media supplements):

Mineral mix (50x):	10 ml
20x Nitrate salts	25 ml
Thiamine 1%	0.5 ml
Sucrose:	171,15 g
Agar	10 g

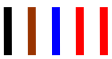
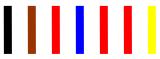
Autoclaved for 20 minutes at 121 °C.

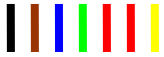
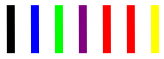
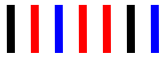
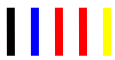
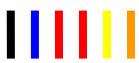
Add media supplements to the Milli-Q H_2O and filter-sterilize.

Table 1. Media overview

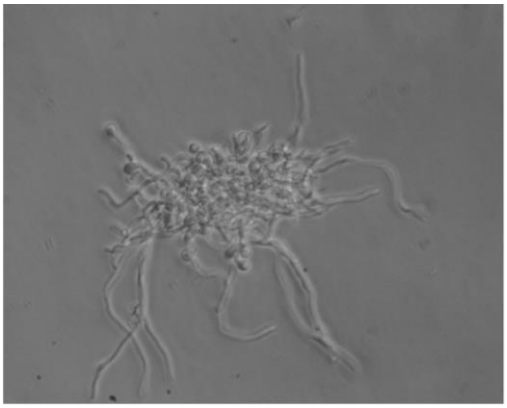
Medium supplements (black + color)								
arginine	solid	4 mM	0.140	0.175	0.35	0.70	g	**arg**
arginine	200 mM	4 mM	4.000	5	10	20	mL	**arg**
methionine	solid	1 mM	0.030	0.0373	0.0745	0.149	g	**met**
methionine	200 mM	1 mM	1.000	1.25	2.5	5	mL	
tryptophane	solid	4 mM	0.163	0.2043	0.4085	0.817	g	**trp**
histidine	200 mM	4 mM	4.000	5	10	20	mL	**his**
threonine								**thr**
uridine	solid	10 mM	0.488	0.61	1.22	2.44	g	**uri**
uracile	solid	10 mM	0.224	0.28	0.56	1.12	g	**ura**
adenine	25 mM	0.5 mM	4.000	5	10	20	mL	**ade**
pyrodoxine	100 µg/mL	0.5 µg/mL	0.100	0.125	0.25	0.5	mL	**pyro**
pantothenic acid		0.008 mM	0.100	0.125	0.25	0.5	mL	**Panto**
riboflavine	200 µg/mL	1.0 µg/mL	0.100	0.125	0.25	0.5	mL	**ribo**
5-fluororotic acid	solid	1.3 mg/mL	0.260	0.325	0.65	1.3	g	**5-FOA**
5-FAA	solid	1.0 mg/ml	0.20	0.25	0.5	1	g	**5-FAA**

Table 2. Overview of media combinations

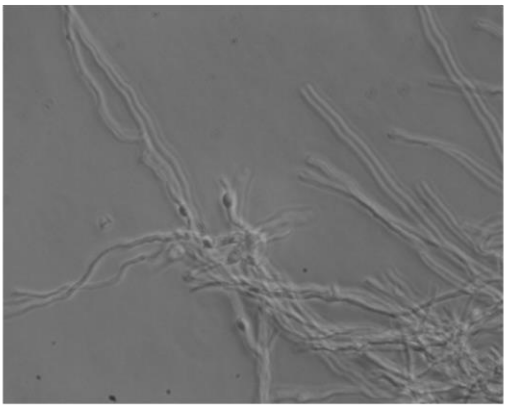
BOX	Compounds in medium	Colour code
18	Minimal medium w.o. supplements (glucose based)	
1	Arg	
2	arg, ura, uri	
3	ura, uri	
4	5-FOA, arg, ura, uri	
5	5-FOA, arg, ura, uri, pyro	
6	ade, arg	
7	ade, arg, pyro	
7	ade, arg, ura, uri, pyro	
9	5-FOA, ade, arg, ura, uri, pyro	
8	arg, pyro	
10	arg, trp	
10	arg, trp, ura, uri	
13	arg, trp, ura, uri, pyro	

13	5-FOA, arg, trp, ura, uri, pyro	
11	trp	
11	ura, uri, trp	
12	arg, trp, 5-FAA	
12	arg, trp, 5-FAA, ura, uri, pyro	
15	arg, ura, uri, met	
14	ade, arg, ura, uri, his	
14	ade, ura, uri, his	
17	arg, ura, uri, met, thr	
9	arg, ura, uri, pyro	
17	arg, ura, uri, panto	
16	ade, ura, uri, panto	
16	ade, arg, panto	
16	arg, ura, uri, pyro, ribo	
18	Minimal medium w.o. supplements (sucrose based)	

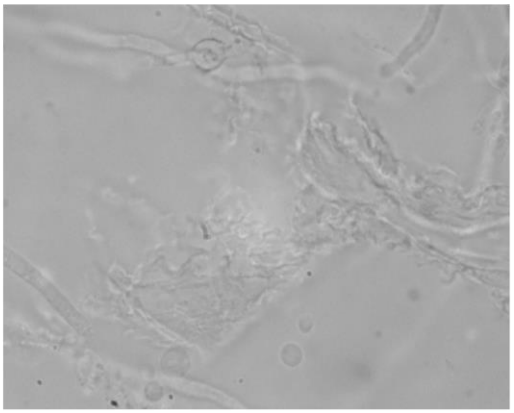
19	Complex medium w.o. supplements	
20	ura, uri, pyro	
21	bio	



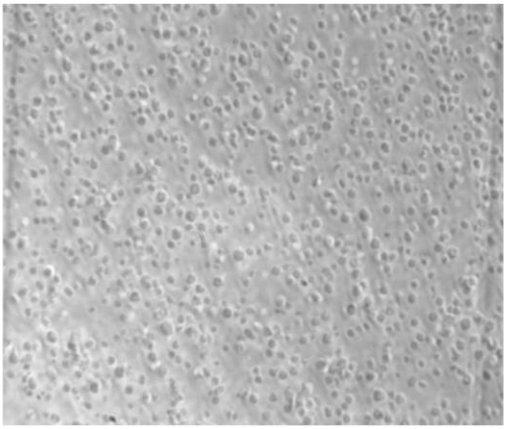
A cluster of IBT 27263 germlings at time zero after adding Glucanex (40x magnified)



The outer boundaries of IBT 27263 mycelium (40x magnified)



Protoplasts mingling with the hyphal remains (100x magnified)



Concentrated protoplasts in a single counting chamber square (40x magnified)