

Comprehensive Guide to Extracting and Expressing Fungal Secondary Metabolites with *Aspergillus fumigatus* as a Case Study

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Fungal secondary metabolites (SMs) have captured the interest of natural products researchers in academia and industry for decades. In recent years, the high rediscovery rate of previously characterized metabolites is making it increasingly difficult to uncover novel compounds. Additionally, the vast majority of fungal SMs reside in genetically intractable fungi or are silent under normal laboratory conditions in genetically tractable fungi. The fungal natural products community has broadly overcome these barriers by altering the physical growth conditions of the fungus and heterologous/homologous expression of biosynthetic gene cluster regulators or proteins. The protocols described here summarize vital methodologies needed when researching SM production in fungi. We also summarize the growth conditions, genetic backgrounds, and extraction protocols for every published SM in *Aspergillus fumigatus*, enabling readers to easily replicate the production of previously characterized SMs. Readers will also be equipped with the tools for developing their own strategy for expressing and extracting SMs from their given fungus or a suitable heterologous model system. © 2021 Wiley Periodicals LLC.

Basic Protocol 1: Making glycerol stocks from spore suspensions

Alternate Protocol 1: Creating glycerol stocks from non-sporulating filamentous fungi

Basic Protocol 2: Activating spore-suspension glycerol stocks

Basic Protocol 3: Extracting secondary metabolites from *Aspergillus* spp grown on solid medium

Alternate Protocol 2: Extracting secondary metabolites from *Aspergillus* spp using ethyl acetate

Alternate Protocol 3: High-volume metabolite extraction using ethyl acetate

Alternate Protocol 4: Extracting secondary metabolites from *Aspergillus* spp in liquid medium

Support Protocol: Creating an overlay culture

Basic Protocol 4: Extracting DNA from filamentous fungi

Basic Protocol 5: Creating a DNA construct with double-joint PCR

Alternate Protocol 5: Creating a DNA construct with yeast recombineering

Basic Protocol 6: Transformation of *Aspergillus* spp

Basic Protocol 7: Co-culturing fungi and bacteria for extraction of secondary metabolites

Keywords: *Aspergillus fumigatus* • fungi • metabolic extraction • natural products • transformation

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INTRODUCTION

Beginning with the discovery of penicillin in 1929 by Alexander Fleming, widespread attention has been given to mining fungal metabolites for pharmaceutical applications (Bennett & Chung, 2001; Keller, Turner, & Bennett, 2005). Secondary metabolites (SMs), which are found in fungi, bacteria, and plants, can be broadly defined as organic compounds not directly involved in the reproduction, growth, or development of an organism (Keller et al., 2005). They often provide important beneficial fitness adaptations that are highly tuned to an organism's ecological niche (Macheleidt et al., 2016). SMs can provide fungi with antimicrobial defenses and protection from ultraviolet (UV) damage, in addition to playing important roles in many developmental structures (Keller, 2019). Out of the 195,000 SMs currently publicly available in a dictionary of natural products (Dictionary of Natural Products, https://dnp.chemnetbase.com/faces/tour_chcd/welcome.xhtml), ~10% are produced by fungi. Out of the SMs with known biological activity, fungi are among the largest sources of antimicrobial, antineoplastic, antiviral, and anti-inflammatory agents (Ntie-Kang & Svozil, 2020). The potential for fungal SM use in medicine, compounded with the estimated 2.2 to 3.8 million fungal species that exist, only 120,000 of which have been characterized, shows strong promise for fungal SM research into the future (Hawksworth & Lücking, 2017).

The methods described in this article detail the entire workflow of growing, transforming, and extracting SMs from *Aspergillus fumigatus*. From start to finish, the user will go from inoculating from a glycerol stock to extracting a metabolic crude sample. Although focused on *A. fumigatus*, the methods can be easily adapted to other fungi, with amendments based on specific growth conditions. Additionally, although it is recommended to use alternative molecular biology methods for the budding-yeast subphylum, the metabolic extraction protocols will be identical. Included are comments, troubleshooting information, and warnings for difficult steps in the protocols, in addition to overall guides for common workflows in SM research. Furthermore, we have created summary methods tables for every characterized SM in *A. fumigatus* (see Table 1 and Supporting Information, Table 1). This should allow readers to quickly reference other laboratories' successful growth and extraction conditions for previously published SMs. This species was chosen because it represents, along with *A. nidulans* (Caesar, Kelleher, & Keller, 2020; Romsdahl & Wang, 2019), the most thoroughly studied filamentous fungus with regard to SM characterization and ecological function of these metabolites (Boysen, Saeed, & Hillmann, 2021; Raffa & Keller, 2019; Wang, Yu, Keller, & Wang, 2021).

Basic Protocols 1 and 2 will explain creating and activating strains from spore-suspension glycerol stocks. These are core techniques for working with sporulating filamentous fungi. Alternate Protocol 1 explains how to create glycerol stocks from non-sporulating filamentous fungi. Basic Protocol 3 and Alternate Protocols 2 to 4 provide four commonly used metabolic extraction techniques. The protocols detail extraction from solid medium or liquid medium with organic solvents that are miscible/immiscible with water, as well as high-volume methods. Basic Protocols 4 to 6 are core methodologies for conducting genetic transformations in filamentous fungi. Basic Protocol 4 details extracting

Table 1 Growth, Conditions, and Extraction Methods for Secondary Metabolites in *A. fumigatus*^a

Metabolite	Strain	Medium	Growth conditions ^b	Method of extraction	Reference(s)
Dihydroxy- phthalene	B-5233 (patient isolate)	AMM (see Supporting Information, Table 1, for recipe)	AMM, 37°C, 5 days	MeOH extraction, followed by 1:1 CH ₂ Cl ₂ /MeOH extraction	Chamilo & Carvalho, 2020; Chiang et al., 2011; Tsai, Wheeler, Chang, & Kwon-Chung, 1999
Endocrocin	Cea17	GMM (see recipe)	Point-inoculate GMM with 1×10^4 spores/ml, 29°C, 10 days	Homogenization and EtOAc extraction	Lim et al., 2012
Ferricrocin	ATCC 46645	GMM (liquid; see recipe)	Inoculate 10^8 conidia into 250 ml GMM, 200 rpm, 37°C, 4 days	Passage through Amberlite XAD-16 resin (see Supporting Information, Table 1, for full details)	Blatzer et al., 2011; Diekmann & Krezdom, 1975; Konetschny-Rapp et al., 1988; Oberegger, Schoeser, Zadra, Abt, & Haas, 2001
Fumagillin	KACC 41191	Czapek-Dox broth (see recipe) + 5 g/L yeast extract (CYA)	CYA, 28°C, 3 days. Move 6-mm-diameter piece to fresh CYA plate, 28°C, 3 days. Again move 6-mm-diameter piece to fresh CYA plate, 28°C, 12 days.	EtOAc extraction	Kang et al., 2013; Lin et al., 2013
Fumigaclavine	Af293	Malt extract agar (see recipe)	Inoculate malt extract agar with 2.5×10^5 spores/ml from spore suspension. Grow for 2-4 weeks.	MeOH extraction	Panaccione & Arnold, 2017
Fumigermin	ATCC 46645	AMM (liquid) for <i>A. fumigatus</i> and M79 (liquid) for <i>Streptomyces rapamycinicus</i>	Grow <i>A. fumigatus</i> pre-culture ~16 hr. Filter with Miracloth. Grow <i>S. rapamycinicus</i> pre-culture on M79 medium. Place mycelium in fresh AMM and inoculate with 1/20 volume of streptomycete culture.	EtOAc extraction	Stroe et al., 2020; Langfelder et al., 1998

(Continued)

Table 1 Growth, Conditions, and Extraction Methods for Secondary Metabolites in *A. fumigatus*^a, continued

Metabolite	Strain	Medium	Growth conditions ^b	Method of extraction	Reference(s)
Fumihopaside A and B	CEA17	PDB (liquid; see recipe)	Grow in 100 ml PDB, 37°C, 4 days	Mycelial isolation and filtering followed by EtOAc extraction (see Supporting Information, Table 1, for full details)	Ma et al., 2019
Fumiquinazol-ine	GA-L7	PDB (liquid; see recipe) + 0.05% (v/v) valproic acid	Incubate 200 rpm, 28°C, 2 days. Inoculate 2% of seed culture into 25 L. Grow at 0.5 vvm, 0.33 bar, 100 rpm, and 28°C for 12 days.	10% MeOH added to broth, followed by DCM extraction	Ames et al., 2011; Magotra et al., 2017; McCloud, 2010
Fumisouquin	Af293	GMM (liquid; see recipe)	Inoculate 1.0 × 10 ⁶ spores/ml in 1 L GMM and grow at 220 rpm, 37°C, 4 days	MeOH extraction	Baccile et al., 2016
Fumitremorgin	BM939	Complete medium	Grow at 28°C, 3-5 days	EtOAc extraction	Kato et al., 2009
Gliotoxin	ATCC 26933	Czapek-Dox broth (see recipe)	Grow at 37°C, 3 days	Chloroform extraction	Dolan et al., 2017; Reeves, Messina, Doyle, & Kavanagh, 2004
Helvolic acid	Marine isolate from authors	PDA and PDB (liquid) (see recipe)	Grow at 28°C, 5 days. Transfer plugs to 300 ml liquid medium. Grow at room temperature, 30 days.	EtOAc extraction, mycelium extraction with acetone followed by EtOAc extraction	Kong et al., 2018
Hexadehydro-astechrome	Af293 & Cea17	GMM (liquid; see recipe)	Inoculate 1.0 × 10 ⁶ spores/ml <i>A. fumigatus</i> and grow at 250 rpm, 25°C, 3 days.	10% MeOH/EtOAc extraction	Yin et al., 2013
Neosartorcin	Heterologous expression in <i>A. nidulans</i>	GMM (liquid; see recipe) + 0.5 μM pyridoxine HCl	Add 10 ⁵ spores per 10 cm (diameter) of plate and grow in the dark at 200 rpm, 2 days	EtOAc/MeOH/AcOH (89:10:1) extraction	Yin et al., 2013
Nidulanin A	Soil isolate from authors	GMM	Inoculate 10 ⁷ spores per 10 cm (diameter) of plate and grow in the dark for 5 days	MeOH extraction, followed by 1:1 CH2Cl2/MeOH extraction	Oakley et al., 2017

(Continued)

Table 1 Growth, Conditions, and Extraction Methods for Secondary Metabolites in *A. fumigatus*^a, continued

Metabolite	Strain	Medium	Growth conditions ^b	Method of extraction	Reference(s)
Pseurotin	Af293	XMM (liquid; 4% xylose)	Pre-culture on MEA medium. Move small agar plugs onto XMM and continue growing at 150 rpm, 30°C, 72 hr.	EtOAc extraction twice, with 6 M HCl added between extractions	Abdelwahed et al., 2020; Yu et al., 2018
Pyomelanin	CEA17	AMM (liquid)	Inoculate 200 ml AMM with 10 ⁷ conidia. Grow at 200 rpm, 37°C, 20 hr. Then, add 10 mM L-tyrosine and grow for 55 hr.	Filter liquid culture through Miracloth and save flow-through	Keller et al., 2011; Schnaler-Ripcke et al., 2009
Pyriptycene A	FO-1289	Custom seed medium and production medium (see Supporting Information, Table 1, for recipe)	Fermentation growth: three rounds of growth and transfer at 27°C (see Supporting Information, Table 1, for full details)	EtOAc extraction	Tomoda, Kim, Nishida, Masuma, & Omura, 1994
Rubrofusarin B ^c	Isolate from authors	PDA and PDB (see recipe)	Three rounds of growth and transfer between PDA and PDB (see Supporting Information, Table 1, for full details)	EtOAc extraction	Hua et al., 2020
Triacetylfusaridine C	Tü 142	Low-iron medium (see Supporting Information, Table 1, for recipe)	Grow at 27°C until extensive conidiation	Passage through Amberlite XAD-16 resin (see Supporting Information, Table 1, for full details)	Blatzer et al., 2011; Diekmann & Krezdorn, 1975; Konetschny-Rapp et al., 1988; Oberreger et al., 2001
Trypacidin	Af293	GMM (see recipe)	Inoculate 5 µl 2 × 10 ⁶ spores/ml spore suspension. Grow in the dark at 29°C, 120 hr.	EtOAc extraction (see Supporting Information, Table 1, for full details)	Throckmorton, Lim, Kontoyannis, Zheng, & Keller, 2016
Xanthocillin	Af293	GMM (see recipe)	Inoculate 10 ml top agar with 1 × 10 ⁶ spores and pour on bottom agar. Incubate at 37°C, 5 days.	EtOAc:MeOH (9:1) extraction	Rafiq et al., 2021

^aThis table condenses information for growing *A. fumigatus* and extracting every characterized secondary metabolite from *A. fumigatus*. This table does not represent a comprehensive list of every possible way to culture said SMs. See Supporting Information, Table 1, for a more detailed summary of the growth and extraction steps, genetic backgrounds, and other pertinent data.

^bGrowth conditions with no temperature listed had none in the cited publications. As such, it is recommend to try using 37°C or 25°C.

^cRubrofusarin B, Alternariol 9-O-methyl ether, Fonsecinone, and Asperpyrone were all grown and collected the same way (Hua et al., 2020).

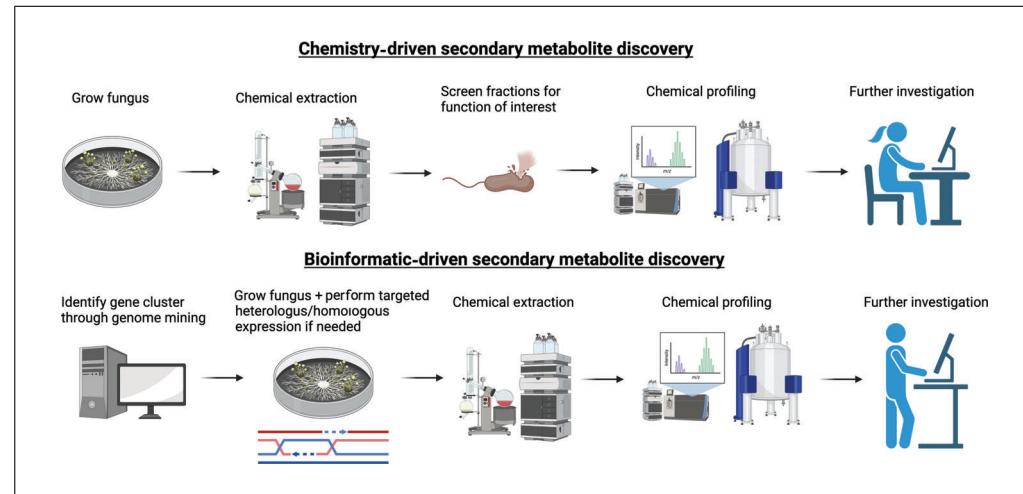


Figure 1 Broad outline of the two common secondary metabolite discovery pipelines. Created with BioRender.com.

genomic DNA, and Basic Protocol 5 and Alternate Protocol 5 explain the methods for creating DNA constructs. Basic Protocol 6 explains the transformation process. Lastly, Basic Protocol 7 details co-culturing methods with bacteria and fungi. For recipes, see Reagents and Solutions, and for tips on troubleshooting, see the step annotations within the protocols, in addition to the troubleshooting table (Table 2) at the end of the article.

STRATEGIC PLANNING

When designing experiments for secondary metabolism studies, much care needs to be taken in choosing the environmental conditions and strains. SM metabolite expression can be strongly influenced by slight changes in medium volume, light, temperature, pH, and nutrients (Keller, 2019). Additionally, different strains of the same species will not always produce the same SMs (Drott et al., 2021).

Although documenting conditions for SM discovery in non-*A. fumigatus* strains goes beyond the scope of this methods paper, most media and growth conditions broadly work for filamentous fungi. Check the literature on your species for commonly used growth conditions and media that work well for your fungus.

There are two commonly seen strategies to studying SMs in fungi, plants, or bacteria (see Fig. 1). The first involves a chemical-based approach, where the researcher identifies the full spectrum of metabolites produced to discover targets of interest. Depending on if the researcher is interested in the function, there may be a screening process to identify fractions from a metabolic extraction that exhibit a given biological activity. Once the fractions of interest have been identified, the compounds must be separated and purified, typically through a series of high-performance liquid chromatography (HPLC) runs. After purification, the compound is structurally identified via a combination of mass spectrometry (MS), UV spectroscopy, crystallography, and/or nuclear magnetic resonance (NMR). This approach was how many of the first natural product discoveries were made in what is sometimes coined as “The Golden Age” of natural product discovery (Katz & Baltz, 2016; Konetschny-Rapp, Huschka, Winkelmann, & Jung, 1988). Today, the strategy is still frequently utilized by more chemistry-oriented labs, where the compounds themselves are the primary focus of research. It is also commonly used on organisms that lack publicly available sequenced genomes and/or are difficult to genetically manipulate. For excellent reviews on fungal identification and compound purification, see the following cited work (Bucar, Wube, & Schmid, 2013; Raja, Miller, Pearce, & Oberlies, 2017; Sticher, 2008).

The second major approach is driven by genome mining and targeted biosynthetic gene cluster (BGC) activation. This is dependent on genomic sequences and gene annotations being available for the fungus of interest. Typically, a BGC will be identified using existing genome-mining software (Chavali & Rhee, 2018; Ren, Shi, & Zhao, 2020). To tie a metabolite to the given BGC, genetic manipulations are required, typically through gene knockouts, overexpression of regulatory factors, and/or manipulation of the epigenome (Keller, 2019). Mutant strains are chemically assessed as above. As more fungal genomes become publicly available, this genome-mining approach can help inform hypotheses on chemical structure and function before any chemical data are obtained, thus allowing for targeted experiments that are more likely to give rise to exciting novel discoveries (Robey, Caesar, Drott, Keller, & Kelleher, 2020). For a summary of the known BGCs and pathway-specific transcription factors in *A. nidulans* and *A. fumigatus*, see Wang, Yu, et al. (2021).

CAUTION: To prevent contamination, always work in a sterile biosafety cabinet/biological safety hood (e.g., Baker SteriGARD III Advance SG403) when working with fungal cultures. Always use sterile equipment and Parafilm your plates to limit accidental contamination. For more sterile best practices, see “I am having frequent contamination issues” in the troubleshooting table (Table 2).

CAUTION: *A. fumigatus*, in addition to other human pathogenic fungi, is a Biosafety Level 2 (BSL2) pathogen. Make sure to follow the proper guidelines at your institution if you are using or handling any BSL2 pathogens.

MAKING GLYCEROL STOCKS FROM SPORE SUSPENSIONS

Glycerol stocks are commonly used in microbiology labs to safely store strains for long periods of time. Unlike bacteria and budding yeast, which are typically grown in liquid medium for glycerol stock creation, filamentous fungal conidia are collected directly from agar plates. Our laboratory commonly uses Glucose Minimal Medium (GMM) for both activation and creation of glycerol stocks; however, any medium that your fungal strain grows on will suffice.

If making glycerol stocks for unicellular fungi such as yeast, the procedure is identical to making glycerol stocks for bacteria; simply substitute the growth medium with an alternative like YPD. For more details, see “Creation of bacterial glycerol stocks” in Basic Protocol 7 or see Basic Protocol 3 in the Current Protocols article by Cosetta & Wolfe (2020).

Common glycerol concentrations in mycology labs range from 33% to 50%. Any value in this range will work well for long-term spore preservation; which you use depends on how viscous you desire your glycerol stock to be.

CAUTION: Take extreme care whenever creating or opening glycerol stocks. It is very easy to have accidental contamination from spores in the air if not working in a very clean environment. Contaminated glycerol stocks can cause serious downstream issues, and contamination can be very difficult to detect. We highly recommend using only sterile filter tips and doing all work in a biosafety cabinet equipped with UV light. When activating multiple strains or creating multiple glycerol stocks, to ensure that cross-contamination does not occur, it is best to turn on the UV light and wait 15 min between working with each strain.

Materials

Plate with growing fungus that has begun sporulation (typically 4 to 5 days old, but this can vary by fungus type)

BASIC PROTOCOL 1

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Sterile 50% (v/v) glycerol (e.g., Fisher, cat. no. 327255000)

Cryogenic tubes (e.g., VWR, cat. no. 89004-320)

Fine-point permanent marker

Sterile cell spreaders (e.g., VWR, cat. no. 76207-748)

Sterile 1000- μ l filter tips (e.g., Phenix, cat. no. TS-059BR)

Freezer storage box

1. Clearly label a cryogenic tube with name of the fungus using a fine-point permanent marker.
2. Take plate of growing fungus that has begun sporulation and place into a sanitized biological safety hood.

Typically, the medium is GMM, but any medium that your fungus sporulates on will work.

3. Add 1 to 2 ml sterile 50% glycerol directly onto plate.
4. Rub spores with a sterile cell spreader until they are suspended in the glycerol solution.

Be careful to not touch any surface with the cell spreader when removing it from the package.

It is helpful to use cell spreaders that have grooves built into them; this makes it easier to disperse the spores from the fungus. Additionally, take care to not use excessive force. If one presses too hard or stirs too vigorously, the mycelium can be broken up and accidentally suspended in glycerol with the spores.

5. Using a sterile 1000- μ l filter tip, put 1 ml spores into labeled cryogenic tube from step 1 and close lid.
6. Repeat for as many strains as need to be stored long term.

If concerned about contamination, this is where you would turn on the UV light and wait ≥ 15 min before starting to work with the subsequent strain.

7. Invert closed cryogenic tubes several times to make sure the spores are properly re-suspended and place into a freezer storage box.
8. Store in a -80°C freezer.

Usually, during the first 24 hr, cell numbers will decrease by 25%. Please re-count the cells after freezing to make sure that you use the correct amount in following experiments (see Basic Protocol 2).

ALTERNATE PROTOCOL 1

CREATING GLYCEROL STOCKS FROM NON-SPORULATING FILAMENTOUS FUNGI

Not every fungal strain will produce spores under laboratory conditions. Additionally, certain mutations prevent sporulation from occurring. When such is the case, it is still important to create glycerol stocks for long-term storage. This protocol is very similar to Basic Protocol 1, except that agar plugs embedded with mycelia are used instead of spore suspensions.

Additional Materials (also see Basic Protocol 1)

Plate with growing fungus that has visible mycelial growth (typically will only take several days of growth but will vary with species and mutations)

Sterile razor blade or equivalent

1. Add 600 μ l sterile 50% glycerol to a cryogenic tube labeled with a fine-point permanent marker.
2. In a sanitized biological safety hood, using a sterile razor blade or equivalent, cut out small agar circles or squares that are embedded with mycelia from a plate with growing fungus that has visible mycelial growth.

Cut small enough pieces to insert about 4 to 5 portions into each cryogenic tube.

3. Place into a freezer storage box and store in a -80°C freezer.

When ready to grow the fungus, thaw and place in liquid medium.

BASIC PROTOCOL 2

ACTIVATING SPORE-SUSPENSION GLYCEROL STOCKS

Assuming the strain is being stored in a spore-suspension glycerol stock (Basic Protocol 1), this is the first important step in extracting SMs from *A. fumigatus* and other sporulating fungi. As with creating glycerol stocks, proper sterile technique is essential to prevent accidental contamination. The medium that is used for the initial activation can be substituted if your strain will grow on it. If applicable, using selective medium can help ensure that only your desired strain grows but may delay the sporulation time.

Materials

Spore-suspension glycerol stock of fungal strain (see Basic Protocol 1)
GMM agar plates (see recipe) or equivalent
Sterile Tween water (see recipe)
70% (v/v) ethanol (EtOH; e.g., Decon Laboratories, cat. no. 64-17-5)

Sterile 200- μ l filter tips (e.g., Fisherbrand SureOne Pipet Tips, cat. no. 02-707-420)
Incubator (e.g., MaxQ 4000, Thermo Scientific cat. no. SHKE4000) at 37°C or
optimal temperature for strain
Sterile cell spreaders (e.g., VWR, cat. no. 76207-748)
25-ml disposable serological pipets (e.g., Dot Scientific, cat. no. 457225)
Motorized pipet filler (e.g., Thermo Scientific S1 Pipet Filler, cat. no. 14-387-165)
50-ml Falcon conical tubes (e.g., Corning, cat. no. 14-432-22)
1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 111564)
Hemocytometer (e.g., Bright-Line, cat. no. Z359629-1EA)
Kimwipes (e.g., Kimberly-Clark, cat. no. 34155)
Glass coverslip
Optical microscope with 10 \times and 40 \times objectives (e.g., Nikon Eclipse E200)

Growing fungus from the glycerol stock

1. Remove spore-suspension glycerol stock of the fungal strain from the -80°C freezer and thaw on ice for roughly 15 to 30 min.
2. Using a sterile 200- μ l filter tip, create three lines on left half of a GMM agar plate or equivalent using 20 μ l of the thawed glycerol stock. Repeat this on right half to make six total streaks.

Slowly and evenly dispense the 20 μ l while making the streaks. This method ensures maximum spore growth with minimal stock being used. An alternative method is to chip a small glycerol fragment from the stock with a sterile toothpick and place it directly onto the medium.
3. Grow strain at 37°C or the optimal temperature for the strain until sporulation has occurred.
4. Place grown fungal plate into a sanitized biological safety hood.

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5. Add 15 to 20 ml sterile Tween water directly onto agar plate.
6. Rub spores with a sterile cell spreader until they are fully suspended in the Tween water.

The same recommendations apply as in the annotation to Basic Protocol 1, step 4.

7. Using a 25-ml disposable serological pipet and a motorized pipet filler, slowly move suspended spores into a 50-ml Falcon conical tube.

Counting the spores using a hemocytometer

8. Dilute spore suspension 10-fold by combining 900 µl Tween water and 100 µl spore suspension in a 1.5-ml microcentrifuge tube.

In order to ensure that the spore suspension and dilutions are homogenized, always vortex before using them. Spores will start to settle on the bottom of the Falcon tube if left alone for several minutes.

9. Further dilute spore suspension 100-fold by combining 900 µl Tween water and 100 µl of the 10-fold dilution in a 1.5-ml microcentrifuge tube. Repeat process to obtain a 1000-fold dilution if the spore concentration is high.
10. Set up a hemocytometer by cleaning it with 70% EtOH and Kimwipes. Place a clean glass coverslip over the hemocytometer.

Moistening the coverslip with water can help affix it to the hemocytometer. If you see Newton's refraction rings, this is an indication of proper adhesion.

11. Gently fill each side of hemocytometer with 10 µl of one of the dilutions (see steps 8 and 9).

Avoid injecting bubbles into the chamber.

The spore suspension will be sucked in by capillary action. It is important to not over- or under-fill the chambers in the hemocytometer. The amount that one should add might vary depending on the brand and type of hemocytometer being used.

12. Place filled hemocytometer under an optical microscope and let sit for 2 min to allow spores to settle.
13. Use 10× objective to localize the view around the larger 3 × 3 grid.
14. Switching to the 40× objective, count all spores inside corner and center squares (see Fig. 2). While counting, use the smaller internal squares as a guide, starting from the top left internal square and snaking down to the bottom right internal square. If the spore touches the left or bottom line, do not count it. If it falls on the right or top line, count it.

We highly recommend using a tally counter (e.g., Fisherbrand Hand Tally Counter, cat. no. 07-905) when doing this work. For increased accuracy, redo the count 1 to 2 times and average among your counts.

The goal is to count anywhere between 25 and 300 spores within the five grids. If you have fewer than 25 spores, use a higher concentration, whereas if you have over 300 spores, use a lower concentration.

15. Clean hemocytometer and coverslip with 70% EtOH and Kimwipes before putting away.
16. To calculate the number of spores per milliliter, use the following equation:

$$\left(\frac{\text{total count}}{5} \right) \times \text{dilution factor} \times 10^4 = \frac{\text{spores}}{\text{ml}}$$

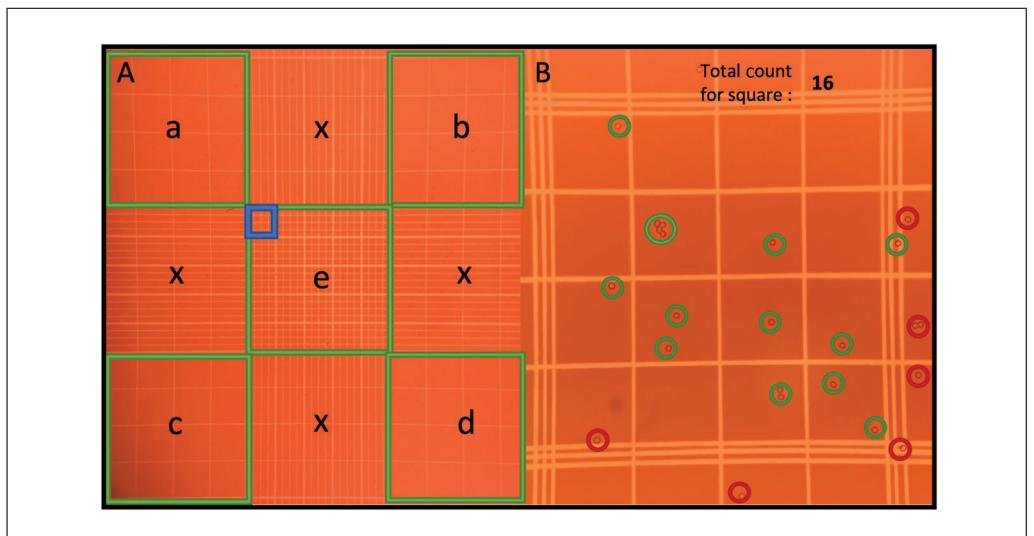


Figure 2 Microscopic view of the hemocytometer through a compound light microscope. **(A)** 100 \times view centered around the large 3 \times 3 grid inside the hemocytometer. Outlined in green are the squares to count. Outlined in blue is what is zoomed in on in **(B)**. **(B)** 400 \times view of the blue square in **(A)**. Circled in green are the spores that would be counted as within the square. Red represents those that are outside of the square.

There are alternative ways to determine total counts that will also work fine. If using an alternative method, make sure to use the correct equation. The large squares in most hemocytometers (assuming that you have the proper coverslip in place) will have a total volume of 0.1 mm³ = 10⁻⁴ cm³, where 1 cm³ = 1 ml.

17. Dilute spore suspension to the desired concentration in Tween water and store spore suspension in a 4°C refrigerator.

Spore suspensions are typically good for ~2 weeks if kept in a 4°C refrigerator. Although they can be kept longer, older spore suspensions are not recommended for use in metabolomic studies.

EXTRACTING SECONDARY METABOLITES FROM *Aspergillus* spp GROWN ON SOLID MEDIUM

BASIC PROTOCOL 3

When extracting SMs from filamentous fungi, the general rule of thumb is that fungi will produce more metabolites when grown on solid medium than in liquid-shake medium (Frisvad, 2012). Additionally, undefined medium that uses vegetative matter (e.g., crushed corn seed, V8, oatmeal; see recipe for oatmeal medium) tends to result in the highest production of metabolites but can lead to more variability. To reduce variability and increase the metabolite extraction yield, it is recommended to first try defined solid medium. If one's metabolite of interest is not produced or is produced in low quantity, try using undefined medium or liquid shake (Frisvad, 2012).

Fungal SM profiles can be altered depending on the concentration of spores added, method of inoculation, growth conditions, medium, and length of time grown (Boruta, 2018). To extract a previously studied SM from the same species, it is recommended to follow previously published conditions as closely as possible (see Table 1).

If studying a new SM, it is recommended to try varying the inoculation method. A point inoculation with a low concentration (10^3) may yield different results than a point inoculation with a higher concentration (10^6 spores/ml). Additionally, one can overlay the agar plates with spores to create a lawn of growth (see Support Protocol).

This basic protocol will detail an extraction using a point inoculation with low concentrations of spores and chloroform. Whereas chloroform is good for extracting organic

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soluble metabolites, alternative solvents must be used to extract water-soluble compounds. Alternate Protocols 2 to 4 detail larger-scale metabolite extractions, extractions from liquid medium, and other commonly used organic solvents. It is important to note that these are not the only ways to extract SMs. Overall, the organic solvent that one should use depends on what dissolves the compound of interest. If the organic solvent is miscible with water, the sample must be lyophilized before the extraction. If the solvent is immiscible, one can separate out the organic and aqueous layers. If using different species, alter the incubation temperature and time to what is most used in the literature.

CAUTION: Chloroform is acutely toxic and should only be worked with while inside a chemical fume hood (e.g., Hamilton Pioneer Fume Hood). Take your time during any steps involving it and always make sure that you are aware of the nearest eye wash station and shower in case accidental skin exposure occurs.

Materials

Molten GMM agar (see recipe) or equivalent
Spore suspension of fungal strain (10^3 spores/ml; see Basic Protocol 2)
Double-distilled water (ddH₂O)
Chloroform (e.g., Fisher, cat. no. C298-4)

100 × 15-mm petri dishes (e.g., VWR, cat. no. 25384-302)
25-ml disposable serological pipets (e.g., Dot Scientific, cat. no. 457225)
Motorized pipet filler (e.g., Thermo Scientific S1 Pipet Filler, cat. no. 14-387-165)
Incubator (e.g., MaxQ 4000, cat. no. SHKE4000) at 28°C or 37°C
5-ml pipet tips (e.g., USA Scientific, cat. no. 1050-0000) or cork borer
Pre-weighed and non-pre-weighed 20-ml glass vials (e.g., Wheaton, cat. no. 03-341-25E)
Homogenizer (e.g., Fisherbrand Homogenizer 150, cat. no. 15-340-168) or spatula
Vortex
Centrifuge (e.g., Thermo Sorvall ST 40R, cat. no. 75-004-525)
Glass Pasteur pipets (e.g., Fisherbrand, cat. no. 13-678-8B)
Scale

Inoculation

1. Add 20 ml molten GMM agar or equivalent to a 100 × 15-mm petri dish with a 25-ml disposable serological pipet and a motorized pipet filler. Once the medium has hardened, inoculate 5 µl spore suspension of fungal strain (10^3 spores/ml) via point inoculation in center of the petri dish.

*If you are unsure about what medium to use, GMM is always a good first choice, especially for the *Aspergillus* spp.*

If you want to utilize a different growth/plating technique (e.g., see Support Protocol), this is the step to change.

2. Incubate in the dark for 3 to 5 days at 37°C if using *A. nidulans* or *A. fumigatus* or at 28°C if using *A. flavus*.

*Although it will take longer to grow, *A. fumigatus* produces more metabolites at 25°C. Altering the temperature to something higher or lower than that at which your species grows quickest can be a way to troubleshoot low yields or a lack of specific SM production.*

Some SMs are growth dependent, meaning that they will only be created at specific stages of the growth cycle. Take care to follow the length of time used in previously published methods if reproducing work.

*Our lab has noted an increase in the production of most metabolites in various *Aspergillus* spp when the fungus is grown in the dark. This is also a variable that should be*

deliberately considered when designing SM experiments. pH and nitrogen/carbon sources also are key components that can induce synthesis of different SMs.

Extraction

3. Remove six plugs (~15 mm in diameter) from GMM plate using a 5-ml pipet tip or a cork borer.

The exact size of the borer is not important. The more plugs you get, the higher the yield but the more time consuming the protocol. We have found 4 to 6 plugs of 15 mm in diameter to be sufficient for most detection methods. If you want to increase the yield, use the entire plate.

4. Place plugs into a 20-ml glass vial.

Using plastic instead of glass will result in some contamination during chloroform treatment. This is fine if running thin-layer chromatography (TLC) procedures, but not for HPLC/MS/NMR procedures.

5. Add 3 to 10 ml ddH₂O to vial and homogenize completely using a homogenizer (usually ~30 s) or break into pieces with a spatula.

The exact amount that you use will depend on the volume of the agar plugs in the glass vial. Avoid filling the glass vial to the brim with water/solvent. When using six 15-mm-diameter plugs in a 20-ml vial, we typically use 7 ml water and organic solvent. Additionally, it is recommended use equal parts organic and aqueous solvents.

6. Add 3 to 10 ml chloroform and vortex for 10 s.

7. Let sample sit for 30 min and shake it every 5 to 10 min.

8. Centrifuge 20 min at 750 × g.

9. Transfer chloroform layer (bottom layer) using a glass Pasteur pipets to a pre-weighed 20-ml glass vial and allow it to evaporate in a chemical fume hood for 2 to 3 days at room temperature.

If trying to speed up the evaporation or working with a volatile compound that may break down if not put into cold storage, bubble nitrogen gas through the extract or use a rotary evaporator.

10. Once dry, using a scale, obtain the new weight of the glass vial. Subtract it from base weight of the vial (see step 9) to obtain the total yield (mg).

11. Store dried extract in a –20°C or –80°C freezer.

When ready for use, resuspend in solvent to the desired concentration in mg/ml.

EXTRACTING SECONDARY METABOLITES FROM *Aspergillus* spp USING ETHYL ACETATE

Chloroform (Basic Protocol 3) and ethyl acetate have similar polarity indexes, meaning that they will extract similar metabolites. However, whereas chloroform is acutely toxic, an irritant, and a suspected carcinogen, ethyl acetate is only a flammable irritant (National Center for Biotechnology Information, 2021a, 2021b). Those with safety concerns about chloroform might opt to use ethyl acetate over chloroform. Broadly, the protocols are the same, the only difference being some steps during the extraction. Follow the inoculation steps in Basic Protocol 3 (steps 1 and 2) before starting this alternate protocol.

Additional Materials (also see Basic Protocol 3)

Ethyl acetate (e.g., Fisher, cat. no. E195-4)

Spatula

Ultrasonic bath sonicator (e.g., VWR, cat. no. 97043-960)

ALTERNATE PROTOCOL 2

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1. Perform steps 1 and 2 of Basic Protocol 3. After 3 to 5 days, remove six plugs (~ 15 mm in diameter) from GMM agar plate using a 5-ml pipet tip or a cork borer.

If scaling up this procedure, you will have to use alternative glassware and a separation funnel during step 4.

2. Place plugs into a 20-ml glass vial and cut into small pieces using a spatula or homogenize with a homogenizer.
3. Add 3 to 10 ml ethyl acetate and sonicate in an ultrasonic bath sonicator for 180 min.

Make sure that you are using glass vials that have lids to prevent unwanted substances from entering your vial during the sonication.

4. Add 3 to 10 ml ddH₂O and shake vigorously for 5 s. Leave upright for 10 min to allow for separation of the two layers.

Sometimes, there will not be a clear separation between the two layers. If this happens, add more ethyl acetate, mix thoroughly, and again let it sit upright until separation occurs.

5. Move ethyl acetate layer (upper layer) to a new pre-weighed 20-ml glass vial to evaporate until dry.

As with the chloroform extraction (Basic Protocol 3), you can speed up the evaporation process with nitrogen gas or a rotary evaporator.

6. Store dried extract in a -20°C or -80°C freezer.

When ready for use, resuspend in solvent to the desired concentration in mg/ml.

ALTERNATE PROTOCOL 3

HIGH-VOLUME METABOLITE EXTRACTION USING ETHYL ACETATE

Sometimes, it is necessary to purify an SM for further chemical characterization, study, or standard use. To obtain larger quantities of the metabolite for purification, it is important to scale up the steps involved in SM extraction. Any extraction method can be scaled up; this example just provides a demonstration of how it would work when extracting from ethyl acetate.

Additional Materials (also see Basic Protocol 3)

ddH₂O

Ethyl acetate (e.g., Fisher, cat. no. E195-4)

Appropriate solvent

Spatula

Blender

2-L beaker

Magnetic stir bar

Magnetic stirrer (e.g., Thermo Scientific Cimarec+, cat. no. S88857108)

50-ml glass serological pipets (e.g., Thomas-Scientific, cat. no. 7543N78)

Rotary evaporator (e.g., Buchi R-100, cat. no. 11100V1001)

Inoculation

1. Perform steps 1 and 2 of Basic Protocol 3.

You can alter the concentration of spores used and the method of plating. See Basic Protocol 3 for more details.

You should have a total of 10 plates per strain being used.

2. Grow plates at 37°C in the dark until the sample has colonized most of each plate.

This is usually around 6 days if growing A. fumigatus at 37°C in the dark.

Extraction

3. Once fully grown, use a spatula to place all agar and fungi from the plates into a blender.
4. Add 250 ml ddH₂O and blend roughly until homogenized (usually only 15 to 30 s).

If using 10 plates and adding 250 ml sterile water, the total volume should be ~500 ml. Scale down/up the amount of water added if using fewer or more plates.

5. Move macerate into a 2-L beaker.
6. Add an equal volume of ethyl acetate and stir vigorously with a magnetic stir bar on a magnetic stirrer for ≥180 min or leave mixture mixing overnight.

For example, if the total volume of the agar and ddH₂O is ~500 ml, add 500 ml ethyl acetate to the beaker.

7. Once 15 min has passed, slow down stirring to allow layer separation.
The stir bar can be removed once the separation is finished.
8. Using a 50-ml glass serological pipet, remove ethyl acetate layer (bottom layer). Evaporate in a rotary evaporator.
Stop short of removing the entire layer to avoid pipetting any unwanted aqueous compounds.

9. Re-dissolve in appropriate solvent and move into a pre-weighed 20-ml glass vial.

The best solvent to use will vary depending on the solubility of your compound in the given organic solvent. If unsure, use the same organic solvent as was used in the metabolite extraction. Common solvents include ethanol, ethyl acetate, methanol, acetone, and acetonitrile.

10. If the total final weight is needed, re-evaporate in glass vial and weigh on a scale. Otherwise, just re-evaporate.
11. Store dried extract in a -20°C or -80°C freezer.

When ready for use, resuspend in solvent to the desired concentration in mg/ml.

EXTRACTING SECONDARY METABOLITES FROM *Aspergillus* spp IN LIQUID MEDIUM

ALTERNATE PROTOCOL 4

This protocol was adapted from Yin, Baccile, et al. (2013), and Yin, Chooi, et al. (2013), who used the method to extract hexadehydroaestochrome from *A. fumigatus*. When using an organic solvent that is miscible with water (i.e., methanol, acetone), the culture should be frozen and lyophilized prior to extraction.

Materials

Spore suspension of fungal strain (10⁶ spores/ml; see Basic Protocol 2)
GMM liquid medium (see recipe) or equivalent
Dry ice
Acetone (e.g., Fisher, cat. no. A18-4)
10% (v/v) methanol (e.g., Grainger, cat. no. 53AZ55) in ethyl acetate (e.g., Fisher, cat. no. E195-4)

2-L Erlenmeyer flask with baffled base (e.g., Corning, cat. no. CLS431281-6EA)
Incubator shaker (e.g., MaxQ 4000, cat. no. SHKE4000) at 25°C
Glass lyophilizer flasks (e.g., Wilmad-LabGlass, cat. no. 14007-774)
Lyophilizer (e.g., Milrock Technology MD53, cat. no. MD3053)

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Magnetic stir bar
Magnetic stirrer (e.g., Thermo Scientific Cimarec+, cat. no. S88857108)
Vacuum filtration or cotton
Pre-weighed 20-ml glass vials (e.g., Wheaton, cat. no. 03-341-25E)
Rotary evaporator (e.g., Buchi R-100, cat. no. 11100V1001; optional)
Scale

Inoculation

1. While in a biological safety hood, add 1 ml spore suspension of fungal strain (10^6 spores/ml) to 1 L GMM liquid medium or equivalent in a 2-L Erlenmeyer flask with a baffled base.

To ensure greater agitation of the culture while mixing (see step 2), it is best to use Erlenmeyer flasks with baffled bases. This leads more consistent growth times between experiments.

2. Grow culture at 25°C while shaking at 250 rpm for 3 days.

Growth time can be sped up by using a temperature closer to 37°C. Ideal temperatures will vary if using a species other than A. fumigatus.

Extraction

3. Place fungal culture (medium and fungal tissue) into a glass lyophilizer flask, freeze in a dry ice/acetone bath, and lyophilize overnight or until the sample has been fully lyophilized.

If you want to extract from the fungal tissue and supernatant independently, they must be separated before lyophilization. Either pour the culture through Miracloth or centrifuge the sample for 15 min at 1900 × g to separate or pellet the fungal tissue. The rest of the extraction protocol will be the same.

4. Add 500 ml of 10% methanol in ethyl acetate to lyophilized tissue. Stir vigorously for 3.5 hr using a magnetic stir bar and magnetic stirrer.
5. Filter extract with vacuum filtration or by pouring over cotton.
6. Put sample into a pre-weighed 20-ml glass vial and evaporate in a fume hood or with a rotary evaporator.
7. Obtain weight of the dried samples using a scale to calculate the yield in mg.
8. Store dried extract in a –20°C or –80°C freezer.

When ready for use, resuspend in solvent to the desired concentration in mg/ml.

SUPPORT PROTOCOL

CREATING AN OVERLAY CULTURE

The following is an alternative method for plating fungal spores (compared to step 1 of Basic Protocol 3 and Alternate Protocols 2 and 3). The result will be a lawn of growth that grows faster than using point inoculation, which consequently speeds up experiments. However, the difference in growth conditions can change the SM profile during the extraction.

Materials

Spore suspension of fungal strain (see Basic Protocol 2)

100 × 15-mm petri dishes (e.g., VWR, cat. no. 25384-302)

25-ml disposable serological pipets (e.g., Dot Scientific, cat. no. 457225)

Motorized pipet filler (e.g., Thermo Scientific S1 Pipet Filler, cat. no. 14-387-165)

Round-bottom flask

Sterile spatulas or equivalent

Additional reagents and equipment for preparing two concentrations of agar (0.7% and 1.5%) in GMM with or without antimicrobial selective agent (see recipe for GMM agar plates)

Option 1: Creating an overlay culture with a sporulating fungus

- 1a. Prepare two concentrations of agar in GMM with or without antimicrobial selective agent.

Typically, 0.7% (w/v) agar is used for the top agar layer, and 1.5% (w/v) for the bottom layer.

- 2a. Add the higher concentration (bottom layer) to 100 × 15-mm petri dishes using 25-ml disposable serological pipets and a motorized pipet filler.

It is important to use the serological pipet and the pipet filler to ensure that the same volume is added to every plate. In our lab, we typically add 15 ml of the 1.5% agar (bottom) layer and 10 ml of the 0.7% agar (top) layer.

- 3a. Calculate how many spores (total) to add to every plate.

Typically, somewhere between 5 and 10 ml top agar is added to each plate.

For example, you want exactly 10⁶ spores to be added to each plate. You are going to add exactly 5 ml top agar; your spore suspension has a concentration of 10⁸ spores/ml, and there are 10 plates in total. Calculations:

$$C \times V = C \times V$$

$$10^8 \text{ spores} \times V (\text{ml}) = 10^6 \text{ spores} \times 5 \text{ ml}$$

$$V = 0.05 \text{ ml} \text{ or } \frac{50 \mu\text{l spore suspension}}{5 \text{ ml top agar}} \text{ or } \frac{10 \mu\text{l spore suspension}}{1 \text{ ml top agar}}$$

$$\begin{aligned} & 10 \text{ plates} \times 5 \text{ ml agar per plate} + 5 \text{ ml for error} \\ & = 55 \text{ ml top agar total} \end{aligned}$$

$$\begin{aligned} & 55 \text{ ml top agar} \times \frac{10 \mu\text{l spore suspension}}{1 \text{ ml top agar}} \\ & = \text{add } 550 \mu\text{l spore suspension to } 55 \text{ ml top agar} \end{aligned}$$

- 4a. Cool down top agar to ~45°C so it is cool to touch but not solid.

If the agar is too hot, it can damage the spores. If it is too cool, it will start to solidify early.

- 5a. Quickly add correct amount of spore suspension (see step 3a), swirl to mix, and plate on top of the solidified bottom agar (see step 2a) with a 25-ml disposable serological pipet motorized pipet filler.
- 6a. Grow culture for 1 to 3 days at the optimal temperature for the species or until the plate is overgrown with mycelium and spores.

As previously mentioned, one can change the growth conditions to a suboptimal growth temperature to potentially increase SM production in the strain.

Option 2: Creating an overlay culture with a non-sporulating fungus

- 1b. Prepare and plate 1.5% bottom layer of GMM agar plates.
- 2b. Grow up small mycelial plugs for 24 to 48 hr in 250 ml GMM liquid medium (liquid shake) in a round-bottom flask at 250 rpm.

If your fungus has a selectable marker, it is recommended to use a selective agent; it can be difficult to maintain sterility in this protocol.

One can scale up or down the total amount of GMM liquid medium to grow the fungus in depending on the scale of the metabolite extraction experiment.

- 3b. Taking care to ensure sterility, grind up fungal balls into a slurry using a sterile spatula or equivalent.

Use a different sterile spatula for each sample.

- 4b. Spread a portion of slurry over GMM agar plates from step 1b to form an overlay culture by pouring out a small amount onto the plate and using a sterile spatula or equivalent.

You only need to spread enough to lightly cover the top of the plates. You do not want there to be a large layer of liquid medium. One “GMM slurry” will be enough to cover many GMM plates.

- 5b. Grow culture for 1 to 3 days at the optimal temperature for the species or until the plate is overgrown with mycelium.

BASIC PROTOCOL 4

EXTRACTING DNA FROM FILAMENTOUS FUNGI

This protocol will detail the method for extracting genomic DNA from *Aspergillus* spp; however, it is routinely used in our lab on *Penicillium* spp and *Alternaria* spp as well. It will work with other filamentous fungi after minor tweaking of the growth conditions. Extracting genomic DNA can be useful for PCR amplification of gene targets, sequencing, and designing constructs for homologous or heterologous expression (Basic Protocol 5). From start to finish, the protocol typically takes 3 to 5 days, with the bulk of that time being the growth and lyophilizing steps.

CAUTION: Phenol is one of the most dangerous compounds commonly found in biology labs. It can cause burns and is absorbed through the skin. Small amounts can be fatal. Always wear proper PPE and only handle it in a chemical fume hood. If some does touch your skin, absorb it with PEG or glycerol before washing with water, as water alone will simply spread the phenol.

CAUTION: Chloroform is acutely toxic and should only be worked with while inside a chemical fume hood (e.g., Hamilton Pioneer Fume Hood). Take your time during any steps involving it and always make sure that you are aware of the nearest eye wash station and shower in case accidental skin exposure occurs.

Materials

GMM liquid medium + 5 g/L yeast extract (see recipe)

Sporulating culture of fungus or spore suspension (see Basic Protocol 2)

Liquid nitrogen

LETS buffer (see recipe)

25:24:1 phenol/chloroform/isoamyl alcohol (e.g., Fisher, cat. no. BP1752I-100)

70% (v/v) and 95% (v/v) EtOH (e.g., Decon Laboratories, cat. no. 64-17-5)

10 mg/ml RNase A (e.g., Qiagen, cat. no. 1007885)

10 mM Tris buffer, pH 8 (see recipe)

100 × 15-mm petri dishes (e.g., VWR, cat. no. 25384-302)

Sterile toothpicks or equivalent

Incubator (e.g., MaxQ 4000, cat. no. SHKE4000) at 37°C

Spatula

Brown paper towels (e.g., Retain, cat. no. 21930) or equivalent

1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 111564)

Glass lyophilizer flasks (e.g., Wilmad-LabGlass, cat. no. 14007-774)

Lyophilizer (e.g., Milrock Technology MD53, cat. no. MD3053)
Microcentrifuge (e.g., Eppendorf Centrifuge 5424, cat. no. 05-400-002), room temperature and 4°C
65°C water bath (Thermo, cat. no. TSGP02)

Additional reagents and equipment for agarose gel electrophoresis (see Current Protocols article; Voytas, 2000)

Inoculation

- Pour 20 ml GMM liquid medium + 5 g/L yeast extract onto a 100 × 15-mm petri dish.

If needed for your strain, make sure that appropriate supplements have been added to the medium. If using 60 × 15-mm petri dishes, add 10 ml medium.

- Using a sterile toothpick or equivalent, pick some *Aspergillus* conidia that have been grown up on solid medium (sporulating culture of fungus or spore suspension) and swirl into petri dish.

It is also possible to use a spore suspension for this inoculation. Add a small amount into the medium and swirl to mix.

- Incubate at 37°C for 16 to 24 hr or until there is a mycelial mat on petri dish that has not begun conidiation.

IMPORTANT NOTE: *It is essential that the culture has not begun conidiation. It becomes very difficult to successfully extract DNA if spores are visible. Additionally, the mycelial mat does not need to cover the entire plate but needs to be large enough to easily move around (taking up at least about one-fourth of the petri dish). If the fungus has not grown enough, check every 30 min until the mycelial mat is sufficiently large.*

Preparing the sample for DNA extraction

- Using a spatula, scoop out mycelial mat and place it onto lid of the petri dish. Ball up mycelium with the spatula and squeeze out as much liquid as possible.

Clean the spatula with 70% EtOH between samples to prevent cross-contamination.

- Transfer mycelial ball to a brown paper towel or equivalent. Fold paper towel and press down on mycelium to squeeze out the remaining liquid. Move it to a dry section of paper towel and repeat until dry.

Typically, the mycelium will be dried after 3 to 5 squeezes on the paper towel.

- Place dried mycelium into a labeled 1.5-ml microcentrifuge tube and poke a hole into the lid.

- Drop mycelium-containing tube into liquid nitrogen.

- Put tube into a cool glass lyophilizer flask when all of the samples are ready. Lyophilize samples overnight or until completely dry.

To cool the glass flask, pour a little bit of the liquid nitrogen into the flask while adding the samples. Let it sit for 1 min to evaporate. If there is excess liquid nitrogen, pour it off before attaching the flask to the lyophilizer.

- Pour lyophilized extract into a different 1.5-ml microcentrifuge tube and break it into a fine powder with a clean sterile toothpick or equivalent.

The sample should not be >100 µl in volume. Pour out excess powder.

If working with many samples, to prevent air moisture-induced aggregation of the DNA through conformational changes (Sharma & Klibanov, 2007), immediately add the LETS

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buffer (see step 10) after breaking up the sample into a fine powder. Then, move on to the next sample.

DNA extraction

10. Add 700 μ l LETS buffer and mix by swirling with the pipet tip. Once partially suspended, fully suspend by inverting tube 10 times. Leave on bench for 5 min to let settle.

11. Move into a chemical fume hood. Transfer supernatant into a new 1.5-ml microcentrifuge tube and add 700 μ l of 25:24:1 phenol/chloroform/isoamyl alcohol. Shake vigorously 30 to 40 times to thoroughly mix. Let sample sit on the bench for 5 min.

CAUTION: Whenever opening the samples from this point on, you must be in the fume hood. The vapor of phenol can be lethal if breathed in for 30 min.

12. Centrifuge samples for 7 min at 9500 $\times g$, 4°C, or until a pellet has formed.

13. Transfer supernatant into a new labeled 1.5-ml microcentrifuge tube and add an equal volume of 25:24:1 phenol/chloroform/isoamyl alcohol. Centrifuge tube for 7 min at 9500 $\times g$, 4°C.

14. Again, move supernatant into a new labeled 1.5-ml microcentrifuge tube and add 1 ml of 95% EtOH.

If needed, this can be a good stopping point; the sample can be left at -20°C overnight after adding the 95% EtOH. Anecdotally, our lab has observed that doing so might help with the quality of the extracted DNA, but this added step is not necessary.

15. Shake sample 10 times and centrifuge tube for 7 min at 9500 $\times g$, 4°C, to pellet DNA.

16. Carefully decant supernatant without losing the pellet.

17. Wash pellet by adding 1 ml of 70% EtOH but do not resuspend the pellet.

This is another good stopping point; the DNA can be stored after adding the 70% EtOH for up to 1 year at -20°C.

18. Centrifuge 2 min at 9500 $\times g$ at room temperature.

19. Once again, decant supernatant and remove excess liquid with a pipet, being careful not to lose DNA pellet. Let it dry at room temperature for 5 to 15 min.

20. Add 20 μ g RNase A (2 μ l from 10 mg/ml stock) to 40 μ l of 10 mM Tris buffer (pH 8) and use solution to resuspend the pellet.

If the pellet is very large, you can add 60 to 100 μ l to decrease the concentration.

21. Incubate samples in a 65°C water bath for 30 min. Tap tubes to mix halfway through.

The incubation will denature any DNase contamination while allowing for RNA degradation.

22. Store at -20°C.

23. Check quality of the genomic DNA by agarose gel electrophoresis (see Current Protocols article; Voytas, 2000).

It is common to see a large band very high up on the gel and a second band that is >10 kb.

CREATING A DNA CONSTRUCT WITH DOUBLE-JOINT PCR

Prior to performing a transformation (Basic Protocol 6), a DNA construct must be created that will be homologously recombined into the genome. For construct creation, double-joint PCR is a quick and cheap method that can be done with common molecular biology equipment (Yu et al., 2004). When deleting a gene, the DNA construct will contain a selectable marker and flanking regions identical to those around the gene of interest. When overexpressing a gene, the DNA construct will contain the gene, a selectable marker, an inducible or constitutive promoter directly in front of the gene of interest's start codon, and the flanking regions. Several reviews detail these strategies (He et al., 2017; Li, Tang, Lin, & Cai, 2017), and the reader is also referred to recent advances using CRISPR-Cas9 (Lim et al., 2021; van Rhijn et al., 2020).

Use of an auxotrophic background strain is common via the deletion of *argB* or *lysB*, which are required for arginine and lysine synthesis, respectively (Xue, Nguyen, Romans, Kontoyiannis, & May, 2004). Auxotrophic background strains exist for many of the commonly studied filamentous fungi. The main antifungal selective agents used in the literature are hygromycin, phleomycin, and pyrithiamine.

When creating strains where multiple rounds of gene deletion/overexpression might be needed, it is recommended to use a self-excising marker. Our laboratory has found great success with the β -Rec/*six* Site-Specific Recombination System, containing the hygromycin resistance gene hygromycin B phosphotransferase (*hph*), as published previously (Hartmann et al., 2010).

The general rules of thumb for good primer design apply for all primer construction steps. Ideally, you want the melting temperature to be around 58° to 60°C. Having a GC clamp on the 5' and 3' ends of the primers helps improve the promoter binding specificity. Avoid repetitive elements and large runs of single nucleotides. See the Internet Resources section of this article for a linked resource that summarizes other do's and don'ts of primer design. The following protocol will show the steps for creating gene-knockout constructs; however, the same general PCR reactions will be universally applicable for other constructs.

NOTE: Experiments involving PCR require extremely careful technique to prevent contamination.

Materials

High-fidelity fusion DNA polymerase and buffer (e.g., PfuUltra II Fusion HS DNA Polymerase and 10× Buffer, Agilent, cat. no. 600672)

Genomic DNA (see Basic Protocol 4)

Gel purification kit (e.g., Qiagen, cat. no. 28706)

Expand Long Template PCR System (Sigma-Aldrich, cat. no. 11 681 842 001)

PCR tubes (e.g., SnapStrip, VWR, cat. no. 490003-692)

PCR machine (e.g., GeneAmp, cat. no. 4339386)

Additional reagents and equipment for primer design, PCR reactions with high-fidelity fusion DNA polymerase (see manufacturer's instructions), and agarose gel electrophoresis (see Current Protocols article; Voytas, 2000)

Primer design

1. Create a marker gene primer set that will amplify selectable marker.

The primers should be 25 to 30 bp long and be on either end of the marker gene (blue arrows; Fig. 3).

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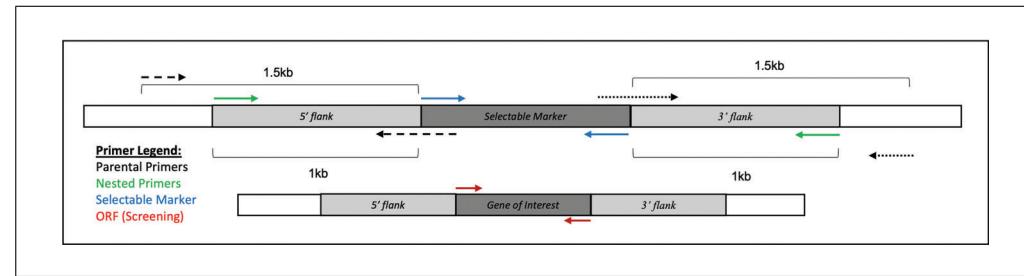


Figure 3 Design for double-joint PCR. Arrows represent primers.

2. Create four primers (include one nested 5' forward primer and one nested 3' reverse primer) that are ~22 bp long.

One primer set should be upstream of the open reading frame (ORF) of the target gene, and the other primer set should be downstream of it. Make one forward primer and one reverse primer anywhere between 1.5 and 2 kb away from the ORF, and make the other set with the nested primer ~1 kb away from the ORF (outer black and green arrows, respectively; Fig. 3).

3. Optional: Create ORF primers for screening (red arrows; Fig. 3) for verifying that transformations were successful in Basic Protocol 6.
4. Create overhang primers for the 3' reverse and 5' forward primers (inner black arrows; Fig. 3).

Add 25 to 30 bp that align with the selectable marker on the 5' or 3' end. The total length will be 52 to 60 bp.

Double-joint PCR

PCR reaction 1

5. Using high-fidelity fusion DNA polymerase and buffer, the correct primers from steps 1, 2, and 4, the genomic DNA from Basic Protocol 4, PCR tubes and a PCR machine, obtain flanking regions and amplify selectable marker.

Run the reactions indicated by blue and black arrows in Figure 4A. To amplify the selectable marker, use the primers from step 1. To amplify the 5' and 3' flanks, use the outer black primers from step 2 and the overhang primers from step 4. For cycling conditions, refer to the manufacturer's instructions for your polymerase system.

6. Run flanks on an agarose gel (see Current Protocols article; Voytas, 2000) to verify successful PCR before moving on (they are needed for step 7). Purify fragments with a gel purification kit.

PCR reaction 2

7. Once again, using high-fidelity fusion DNA polymerase and buffer, mix amplicons from step 6 at a ratio of 1:3:1 5'-flanking/selectable marker/3'-flanking regions in PCR tubes. Run 10 cycles on a PCR machine, ensuring that annealing temperature is held long enough for the entire construct (Fig. 4B).

The exact hold time for the annealing step will vary depending on the enzyme used. A general rule of thumb is 1 min per kb of your construct. All other cycling steps should follow the manufacturer's instructions.

This reaction creates the full DNA construct using the overhang regions as primers for each polymerase reaction.

PCR reaction 3

8. Use 2 to 4 ml from step 7 and Expand Long Template PCR System to amplify the entire DNA construct using the nested primers from step 2 (Fig. 4C) in PCR tubes.

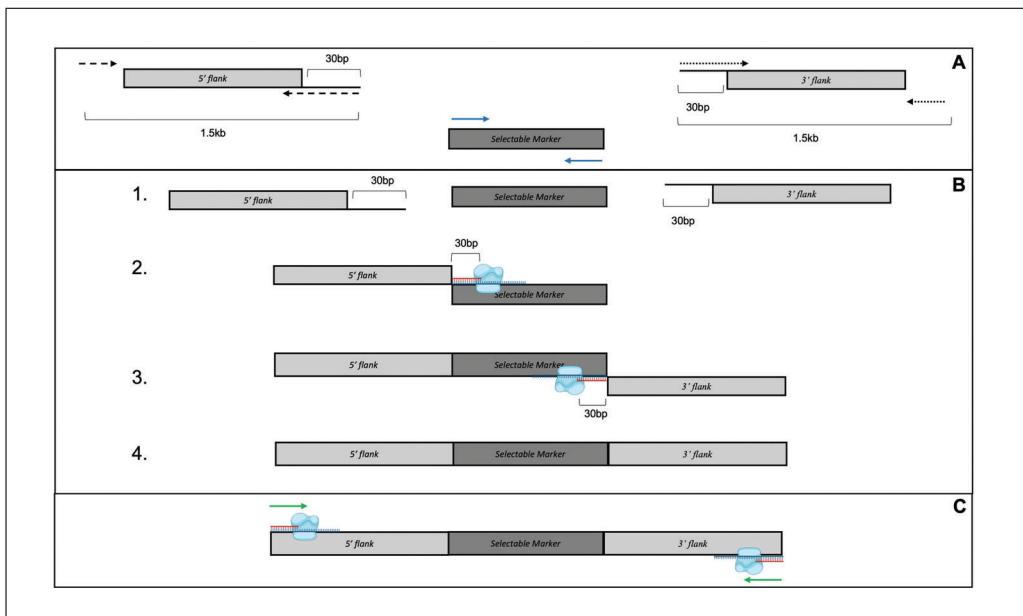


Figure 4 The three rounds of PCR when performing a double-joint PCR reaction. **(A)** PCR reaction 1 will amplify the three fragments. **(B)** PCR reaction 2 uses the overhang region to serve as the primer for the DNA polymerase, joining together the three fragments. **(C)** PCR reaction 3 amplifies the DNA construct that was created in reaction 2.

The total volume should be 100 μ l. Follow the manufacturer's instructions for cycling conditions.

The Expand Long Template PCR System does not have surfactants. This is very important, as surfactants can cause protoplast rupturing during the transformation. If using an alternative polymerase, ensure that no surfactant is included in the buffer.

- Verify that PCR amplification worked by running on an agarose gel (see Current Protocols article; Voytas, 2000).

One can optionally purify the DNA construct after this step instead of purifying it during the transformation (see step 6) with a G-50 column (see Basic Protocol 6, steps 18 and 19).

CREATING A DNA CONSTRUCT WITH YEAST RECOMBINEERING

Double-joint PCR (Basic Protocol 5) is a cheap and relatively easy technique for synthesizing DNA constructs. However, it starts to become less effective when the DNA construct is very large (>10 kb). This is often the case when transferring entire gene clusters into a fungus for homologous expression. When such is the case, you can transform yeast competent cells with overlapping DNA fragments to have the yeast “stitch” them together. Although most fungi favor non-homologous end-joining (NHEJ) over homologous recombination (HR) to resolve DNA damage, budding yeast such as *Saccharomyces cerevisiae* efficiently use HR as their primary pathway (Gardner & Jaspersen, 2014; Zhang et al., 2011). This protocol requires the use of a yeast and an *Escherichia coli* shuttle vector. Any equivalents to the ones that we use will work fine for this method.

NOTE: Experiments involving PCR require extremely careful technique to prevent contamination.

Materials

- Yeast competent cells (e.g., Thermo MaV203 Competent Yeast Cells, cat. no. 11281011)
- 2× YPAD medium (see recipe)

ALTERNATE PROTOCOL 5

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ddH₂O
Sterile frozen competent cell (FCC) solution: 5% (v/v) glycerol (e.g., Fisher, cat. no. 327255000) and 10% (v/v) dimethyl sulfoxide (DMSO; e.g., Sigma-Aldrich, cat. no. D8418)
50% (w/v) PEG 4000 or 6000 (e.g., Calbiochem, cat. no. 528877)
1.0 M lithium acetate (LiAc), pH 7.5 (e.g., Fisher, cat. no. AC268640010)
2 mg/ml single-stranded carrier DNA (e.g., salmon sperm DNA, Sigma, cat. no. D-1626)
Digested pE-YA plasmid vector (Pahirulzaman, Williams, & Lazarus, 2012) or equivalent
PCR products (DNA fragments generated by completing up to step 5 of Basic Protocol 5)
Appropriate selective medium with agar (for transformed yeast cells)
Appropriate selective liquid medium (for transformed yeast cells)
Miniprep plasmid purification kit (e.g., Bio-Rad, cat. no. 732-6100I)
E. coli competent cells (e.g., Thermo DH5 α Competent Cells, cat. no. 18265017)
LB liquid medium (see recipe)
LB agar plates (see recipe) with selective agent
Incubator shaker (e.g., MaxQ 4000, cat. no. SHKE4000) at 30°C
125-ml Erlenmeyer flask
Sterile 500-ml Erlenmeyer flask with baffled base (e.g., Corning, cat. no. CLS431401)
OD reader (e.g., Thermo SPECTRONIC) or hemocytometer (e.g., Bright-Line, cat. no. Z359629-1EA) with glass coverslip
50-ml Falcon conical tubes (e.g., Corning, cat. no. 14-432-22)
Centrifuge (e.g., Thermo Sorvall ST 40R, cat. no. 75-004-525)
1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 111564)
Microcentrifuge (e.g., Eppendorf Centrifuge 5424, cat. no. 05-400-002)
Vortex
37°C and 42°C water baths (e.g., Thermo, cat. no. TSGP02)
Sterile spatula
Round-bottom culture tubes (e.g., Falcon, cat. no. 9217F80)

Additional reagents and equipment for colony PCR (see Current Protocols article; Woodman, Savage, Arnold, & Stevenson, 2018)

Preparing frozen yeast competent cells

1. Inoculate yeast competent cells into 25 to 50 ml of 2 \times YPAD medium in a 125-ml Erlenmeyer flask. Incubate overnight at 30°C while shaking at 200 rpm.

Most yeast shuttle vectors will work. If YPAD does not make sense for your strain, use SD with the appropriate supplements.

One does not need to create frozen yeast competent cells (steps 1 to 13) every time the protocol is run. As written, the protocol yields 50 tubes of competent cells, enough for 50 transformations. Make the necessary adjustments based on your requirements.

2. Prepare a sterile 500-ml Erlenmeyer flask with a baffled base by filling it with 250 ml of 2 \times YPAD.
Leave the flask capped at room temperature. Alternatively, you can prepare this after determining the concentration in step 4; it will be used for step 5.
3. Incubate culture overnight at 30°C while shaking at 200 rpm.
4. Determine concentration of the culture using an OD reader or a hemocytometer with a glass coverslip, calculate volume of culture that would contain 1.25×10^9 cells,

and move it into its own 50-ml Falcon conical tube. Centrifuge 5 min at $3000 \times g$ or until pelleted.

For OD₆₀₀ reading of the culture, set 1×10^7 cells/ml to an OD reading of 1. To obtain the concentration using a hemocytometer, see “Counting the spores using a hemocytometer” in Basic Protocol 2.

5. Pour off supernatant and resuspend cells with fresh medium from the pre-filled 500-ml Erlenmeyer flask containing 250 ml of 2× YPAD from step 2. Once suspended, pour cells into the 500-ml flask.

If you had 1.25×10^9 cells pelleted and resuspended them in 250 ml, the final concentration would be 5×10^6 cells/ml.

6. Incubate flask at 30°C while shaking at 200 rpm until the density has reached 2×10^7 cells/ml.

This allows the cells enough time to undergo two divisions, entering the early log phase.

7. When ready, centrifuge cells for 5 min at $3000 \times g$ in 50-ml Falcon conical tubes.
8. Pour off YPAD supernatant and resuspend pellet in 25 ml ddH₂O. Transfer to a single 50-ml Falcon conical tube.

9. Wash cells by centrifuging for 5 min at $3000 \times g$. Decant supernatant.

10. Resuspend cells in 25 ml ddH₂O. Repeat step 9 to perform a second ddH₂O wash.

11. Resuspend pellet in sterile FCC solution to a concentration of 2.0×10^9 cells/ml.

12. Dispense 50 µl cell solution into 1.5-ml microcentrifuge tubes.

This means that there should be 1×10^8 cells in each tube.

13. Slow-freeze cells by placing them in a -20°C freezer and moving them into a -80°C freezer after 3 hr.

The cells can be kept in the -80°C freezer for long-term storage.

Transforming competent yeast

14. Thaw as many frozen cells as you need.

15. Centrifuge 2 min at $13,000 \times g$ and remove supernatant.

16. Add the following directly to the cell pellet as listed: 250 µl of 50% PEG 4000 or 6000, 36 µl of 1.0 M LiAc (pH 7.5), and 50 µl of 2 mg/ml single-stranded carrier DNA.

17. Create DNA master mix by adding 250 ng digested pE-YA plasmid vector or equivalent and 500 ng of each PCR product. Bring up final volume to 14 µl with ddH₂O.

18. Vortex until fully resuspended.

19. Incubate in a 42°C water bath for 45 min.

It can help to mix periodically by gently inverting the tubes.

20. Centrifuge tubes for 30 s at $13,000 \times g$ and remove supernatant.

21. Resuspend pellet in 1 ml ddH₂O and gently resuspend by pipetting.

Avoid vortexing.

22. Plate out 200 to 500 µl onto appropriate selective medium with agar.

The goal is to get several hundred transformants. The appropriate selective medium will depend on the selectable marker on the plasmid vector being used. An example medium that would work is SD + the selectable marker.

23. Incubate at 30°C for 3 to 5 days.

Plasmid purification from yeast

24. Using a sterile spatula, scrape colonies into a round-bottom culture tube containing 5 ml of appropriate selective liquid medium.

Use a liquid form of the appropriate selective medium that was used in step 22.

25. Incubate overnight at 30°C while shaking at 200 rpm.

26. Using two 1.5-ml microcentrifuge tubes per 5 ml of culture, spin down 1.25 ml in each tube for 20 s at max speed. Pour off supernatant. Repeat another time in same tubes to end up with 2.5 ml of culture having been spun down in each tube.

27. Use a miniprep plasmid purification kit to purify plasmids from the yeast competent cells.

Transforming into competent *E. coli*

28. Thaw *E. coli* competent cells on ice.

You will need 100 µl cells per tube.

29. Add 5 to 10 ng purified plasmid to labeled 1.5-ml microcentrifuge tubes.

30. Once the cells are thawed, add 100 µl of the competent *E. coli* to each tube. Flick to mix and place on ice for 30 min.

31. Remove from ice and heat-shock cells for 2 min by placing them in a 37°C water bath.

32. Add 900 µl LB liquid medium and incubate in 37°C water bath for 30 min.

33. Plate out two dilutions of transformation mixture by spreading 10 µl and 100 µl onto LB agar plates with selective agent. Let plates sit upside down for 5 min before turning them over.

34. Incubate at 37°C overnight or until colonies are at the desired size.

35. Pick colonies and screen for desired vector via colony PCR (see Current Protocols article; Woodman et al., 2018).

There are other screening techniques that would be sufficient here (e.g., restriction digests, sequencing).

36. Once confirmed, purify plasmid with a miniprep plasmid purification kit.

*It is recommended to make a glycerol stock of this *E. coli* strain once the isolate is confirmed by PCR; this will prevent you from having to redo this protocol if you need more of the DNA construct in the future.*

BASIC PROTOCOL 6

TRANSFORMATION OF *Aspergillus* spp

Transformation of filamentous fungi allows for overexpression and deletion of genes or entire gene clusters. This protocol describes how to transform filamentous fungi with a DNA construct (Basic Protocol 5 and Alternate Protocol 5) via homologous recombination.

After transformation, DNA must be extracted from all transformants for confirmation of successful DNA construct insertion (Basic Protocol 4). Verification of the transformation

typically involves PCR screening, Southern blot analysis, or sequencing (Stroe et al., 2020; Southern, 2006; Wang, Drott, et al., 2021).

The negative control (most important) should typically be a sample that does not receive the DNA construct, and the positive control is a few micrograms of DNA known to transform well. The negative control is essential to check for contamination that grows on the selective GMM. The positive control allows you to determine if the cells were able to transform at all. Failure of the positive control to yield transformants is a sign that there is something wrong with your methodology or reagents.

Materials

Plates containing grown fungus (see Basic Protocol 2)
Liquid Minimal Medium (LMM; with or without yeast extract; see recipe)
Sterile ddH₂O
Lysing enzymes (e.g., Sigma Lysing Enzymes, Trichoderma, cat. no. L1412-25G)
Yatalase (e.g., TaKaRa, cat. no. SD5214)
Osmotic medium (see recipe)
Trapping buffer (see recipe)
STC buffer (see recipe)
DNA construct to transform (see Basic Protocol 5)
10 mM Tris buffer, pH 8 (see recipe)
PEG solution (see recipe)
SMM bottom and top agar (with selective agent; see recipe for SMM agar plates and Support Protocol, step 1a)
GMM agar plates (with selective agent; see recipe)

125-ml and 1-L Erlenmeyer flasks
Incubator shaker (e.g., MaxQ 4000, cat. no. SHKE4000) at 15°C, 27°C, and 37°C
50- and 250-ml centrifugation bottles (e.g., Nalgene, cat. no. cat. no. 3119-0050PK and 05-579-20)
Centrifuge (e.g., Thermo Sorvall ST 40R, cat. no. 75-004-525, with TX-750 rotor), 20°C
0.45-μm syringe filter (e.g., Grainger, cat. no. 12K961)
Sterile spatula
50-ml Falcon conical tubes (e.g., Corning, cat. no. 14-432-22)
25-ml disposable serological pipets (e.g., Dot Scientific, cat. no. 457225)
15-ml centrifuge tubes (e.g., Fisher, cat. no. 05-539-4)
1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 111564)
Microcentrifuge (e.g., Eppendorf Centrifuge 5424, cat. no. 05-400-002)
G-50 column and G-50 matrix (e.g., Cytiva G-50 Columns, cat. no. 27533001; optional)
Motorized pipet filler (e.g., Thermo Scientific S1 Pipet Filler, cat. no. 14-387-165; optional)

Additional reagents and equipment for spore harvesting (see Basic Protocol 2, steps 4 to 7), determining spore concentration (see Basic Protocol 2, steps 8 to 17), point inoculation and incubation (see Basic Protocol 3, steps 1 and 2), and preparing glycerol stocks of transformants (see Basic Protocol 1)

Inoculation

1. Harvest spores from at least four plates containing grown fungus per desired transformant background (see Basic Protocol 2, steps 4 to 7) and determine spore concentration with a hemocytometer (see Basic Protocol 2, steps 8 to 17).

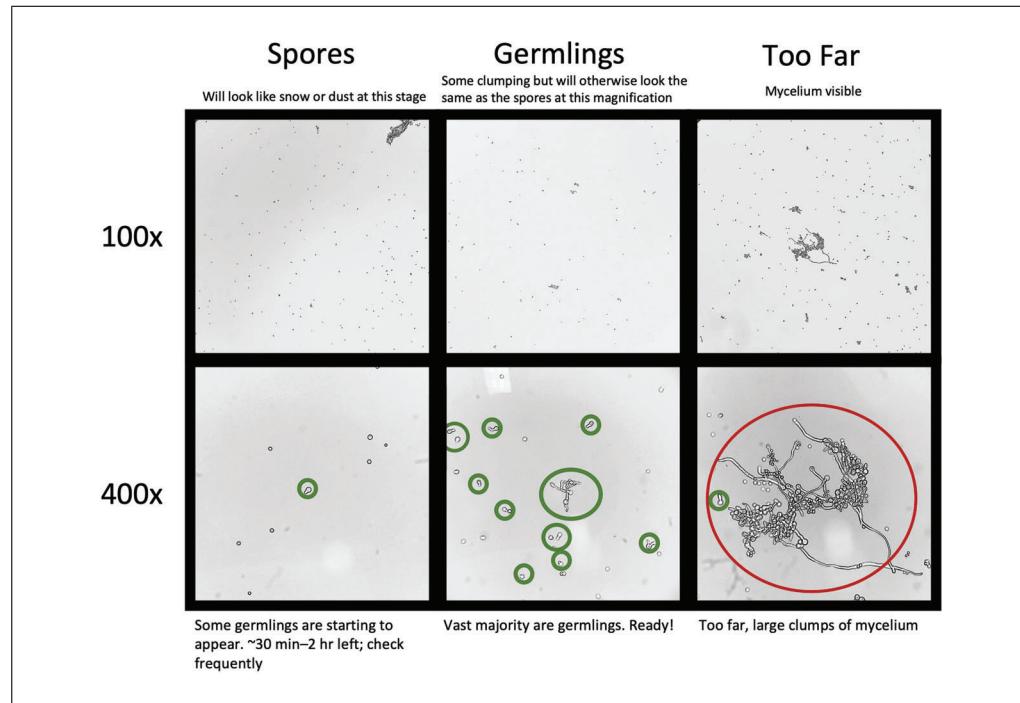


Figure 5 Example images of microscopic slides when checking for germlings in *P. expansum*. Circled in green are germlings. Circled in red is a clumping of mycelium. You want a majority of the cells to be germlings, with a few ungerminated spores. If you are seeing mycelial growth clumping together, the culture has grown too long.

2. Inoculate 500 ml LMM (add supplements if appropriate) with 10^9 spores in a 1-L Erlenmeyer flask and incubate at 27°C while shaking at 250 rpm for approximately 10 to 12 hr if yeast extract was added or 12 to 15 hr if yeast extract was not added (assuming *A. fumigatus* use). Alternatively, grow culture at 37°C while shaking at 250 rpm for 5 to 9 hr.

A culture that is ready for step 3 will have mostly young germlings that typically clump together in small aggregates (see Fig. 5).

This is the trickiest part of the transformation, as the exact timing can vary between experiments, strains, and minor condition changes. A proper culture will have mostly young germlings in small aggregates (see Fig. 5). Young germlings are larger than spores but will not have mycelial growth. If you check and there are some young germlings but also a fair number of spores, leave the culture to grow and check every 30 min until there is an abundance of aggregated germlings.

*When running a transformation with *Penicillium expansum*, we found incubating an initial culture of 250 ml with 10^9 spores in 250 ml LMM + yeast extract for 10 hr at 25°C, 280 rpm, to be ideal. The conditions will be unique for each species.*

If the culture has overgrown and the germlings have begun growing hyphae, do not continue with the rest of the experiment; it will be impossible to create protoplasts. Simply start over with inoculating an LMM culture with the spore suspension.

Making protoplasts

3. Transfer culture into 250-ml centrifugation bottles and centrifuge 2 min at 11,500 $\times g$, 20°C.

Alternatively, steps 3 to 6 can be replaced with filtering the culture through sterile Miracloth, followed by washing the mycelia with 50 ml mycelium wash (see recipe).

4. Decant supernatant, taking care to not lose any germlings, and resuspend in 50 ml sterile ddH₂O.

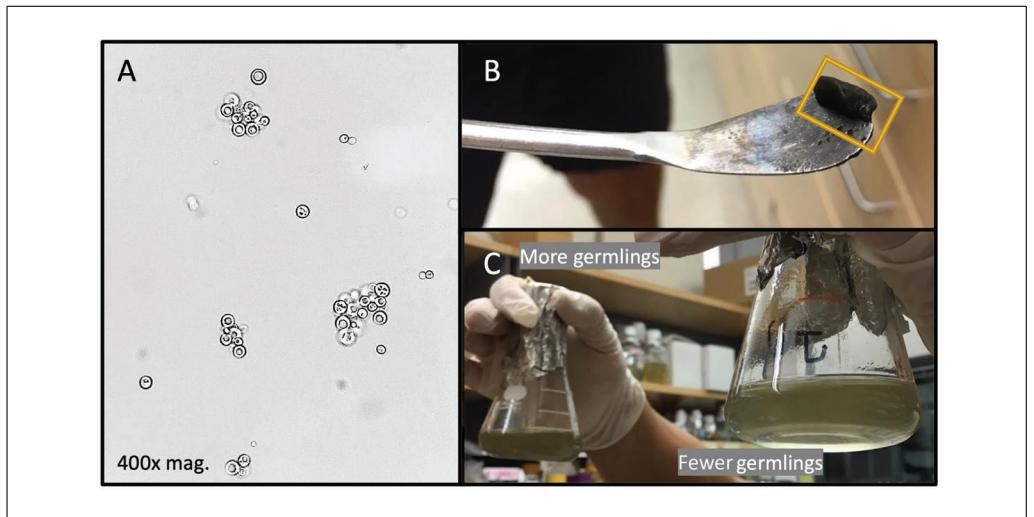


Figure 6 **(A)** Image of protoplasts under a microscope at 400 \times . A protoplast will appear larger than a spore, and the vacuole inside of the cell will be visible. Clumping is normal. The sample is ready when the vast majority of germlings are protoplasts under a microscope. **(B)** Example of the proper amount of germlings that one should add during step 8 of Basic Protocol 6. Any amount from this amount to the yellow box would be acceptable. Avoid over-adding, or you will have difficulty making the protoplasts. **(C)** It is common practice in our lab to create two flasks during step 8 of Basic Protocol 6. Mix more germlings into one and fewer into the other; this should be visible, as the former flask will have a darker color.

5. Transfer to a 50-ml centrifugation bottle and centrifuge 1 min at 25,000 \times g, 20°C. Decant supernatant.
 6. Repeat wash steps (steps 4 and 5) one more time.
 7. Mix 90 mg lysing enzymes, 60 mg Yatalase, and 30 ml osmotic medium. Filter through a 0.45- μ m syringe filter into a 125-ml Erlenmeyer flask.
- Optional: Prepare two Erlenmeyer flasks to test two different concentrations of germlings during step 8.*
8. Move a tipful of the germlings into the flask with a sterile spatula (see Fig. 6B).
- Add anywhere from the amount shown in Figure 6B to two times that amount (stay roughly within the drawn yellow box if adding more). If inoculating two flasks, it is recommended to use slightly different amounts in each one (see Fig. 6C). This makes it more likely that at least one will work. The ideal time for protoplasting will vary between the two flasks.*
9. Shake at 100 rpm (max 120 rpm) for ~4 hr at the same temperature as in step 2. Check periodically, starting at 2-hr timepoint, under an optical microscope to verify that the protoplasts are forming (Fig. 6A). Remove from shaker if there is an abundance of protoplasts in the culture.

*This is another step that can vary wildly depending on the species/strain. It also is very sensitive to the number of germlings added in step 8. As an example, *A. fumigatus* tends to take longer than *A. flavus* or *P. expansum*. Check frequently if running for the first time and carefully document when protoplasts formed from your background strain. This will give you better estimates for how long this step will take if using similar background strains in the future.*

10. Pour cells directly into a 50-ml Falcon conical tube. Pipet up and down several times with a 25-ml disposable serological pipet.

Avoid pipetting vigorously but provide enough force to mechanically break up protoplast clumps and debris.

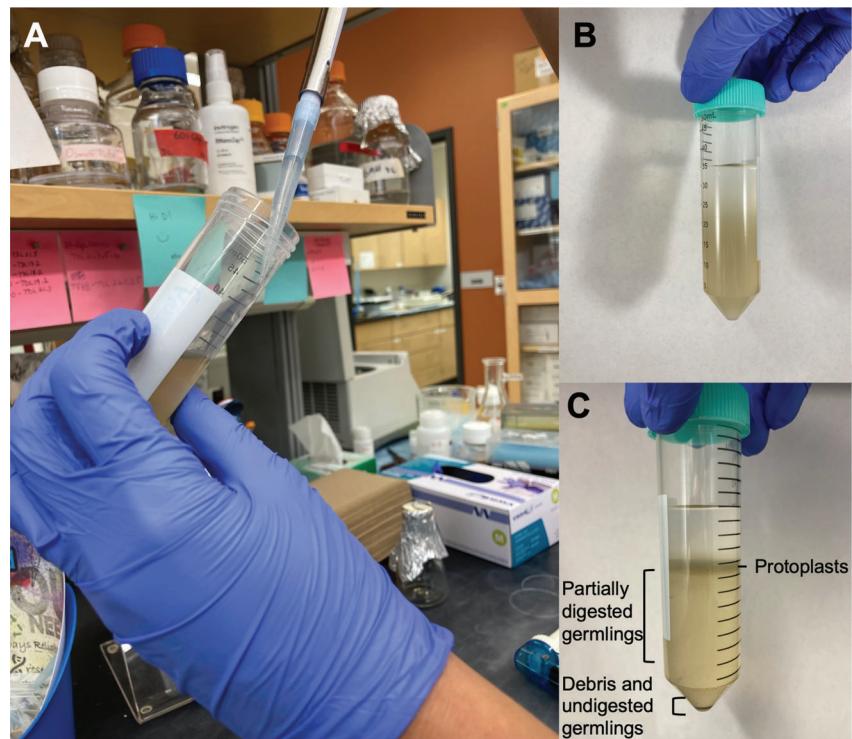


Figure 7 Images of the trapping buffer in steps 11 and 12 of Basic Protocol 6. **(A)** Angling the 50-ml conical tube so that the trapping buffer slowly runs along the wall. **(B)** Mixture before centrifugation. Top layer: Trapping buffer. Bottom layer: Culture. **(C)** Mixture after centrifugation. Protoplasts will be trapped in the interface, as labeled on the image.

11. Hold tube at an angle and very slowly add 4 ml trapping buffer with a P1000 tip (Fig. 7A).

If dispensing slowly enough, two layers should start to form (Fig. 7B), with the protoplasts being trapped in the middle. Holding the tube at an angle helps in dispensing the liquid on the wall of the tube.

12. Centrifuge 15 min at $1300 \times g$, 20°C.
13. Using a P1000 tip, pipet protoplasts from the interface (Fig. 7C) and transfer to a 15-ml centrifuge tube.

If you have a total of ≤ 3 ml, use a single centrifuge tube. Otherwise, split the protoplasts into two separate tubes.

14. Add STC buffer to maximum volume and invert 8 to 10 times. Let sit on ice for 2 to 3 min.

The STC buffer does not need to be pre-chilled while running the transformation; simply remove it from the 4°C refrigerator when starting the transformation and place it back into the refrigerator when done using it.

The osmotic medium contains MgSO₄, which makes the protoplasts float. To properly dilute it, the STC volume needs to be at least 2.5-fold the volume of the osmotic medium. Diluting the MgSO₄ allows vacuoles to leave the cells, making them sink.

15. Optional: If there appears to be a lot of debris (partially digested protoplasts and spores), centrifuge 1 min at $200 \times g$ (1000 rpm with TX-750 rotor), 20°C. Incubate on ice for 5 min and transfer supernatant to another 15-ml centrifuge tube.

The mixture will appear darker if there are lots of spores and partially digested protoplasts. The pellet is the debris, and the protoplasts will be left in the supernatant. Do NOT centrifuge faster, or the pellet will be debris and protoplasts.

16. Centrifuge 7 min at $7800 \times g$, 15°C, to collect protoplasts. Decant supernatant and resuspend by tapping in 1 ml STC buffer. Move into a 1.5-ml microcentrifuge tube.
17. Centrifuge 15 s at max speed in a microcentrifuge. Resuspend in $\geq 200 \mu\text{l}$ STC buffer per construct being transformed.

For example, if you have two constructs, you need $2 \times 200 \mu\text{l} = 400 \mu\text{l}$ STC buffer. Add 50 μl to account for pipetting error (450 μl total).

Recommended: Determine the concentration using a hemocytometer. Save the dilution to calculate the total viable protoplasts. For each construct that is being transformed into the cells, you want to have $\sim 100 \mu\text{l}$ of 10^8 protoplasts per milliliter.

If the concentration of the protoplasts is $< 5 \times 10^6/\text{ml}$, it is unlikely that the transformation will work.

Purifying the DNA construct with a G-50 column (skip if construct has already been purified)

18. Fill a G-50 column with G-50 matrix and centrifuge 2 min at $600 \times g$. Decant flow-through and place into a clean 1.5-ml microcentrifuge tube.

Steps 18 and 19 are not needed if the DNA construct has already been purified.

19. Transfer DNA construct to the G-50 column. Centrifuge column for 2 min at $600 \times g$. Save flow-through.

Transformation

20. In a clean labeled 1.5-ml microcentrifuge tube, combine 20 to 25 μl of the G-50-purified construct and complete to 50 μl with 10 mM Tris buffer (pH 8). Add 50 μl STC buffer.

The total volume should be 100 μl .

21. Add 100 μl protoplast solution from step 17, tap to mix, and incubate on ice for 50 min.
22. Add 1.25 ml PEG solution. Mix gently by turning and rotating tube. Incubate at room temperature for 15 to 20 min.

Do not let it sit for longer than 20 min. While the exact mechanism of PEG is only hypothesized, it is thought to help bring the DNA construct closer to the membrane by altering the water structure around plasma membranes (O'Connor, 2013). Leaving the solution sitting for too long can be harmful to the protoplasts.

23. Add 5 ml STC buffer and mix by inversion. Place on ice and keep on ice when not using.

Optional: You can place the protoplasts in a 4°C refrigerator overnight to increase their efficiency.

24. Add 6 ml protoplast solution to cooled SMM top agar (containing selective agent) and swirl to mix.

This SMM top and bottom agar must contain your selective agent. You need at least six plates per transformation.

Alternatively, you can sandwich 500 μl to 1 ml protoplast suspension between the GMM bottom and top agar. First, plate the protoplast suspension on bottom agar and incubate at room temperature overnight right side up to dry. Then, add top agar.

25. Gently pour SMM top agar or use a motorized pipet filler to spread it.
26. Incubate at 37°C until small colonies have formed (2 to 3 days for *A. fumigatus*).

Some mutations will impact the fungus's ability to grow and thus it may take longer.
27. Transfer the small isolates to their own GMM agar plate (containing correct selective agent) via point inoculation and incubate until conidiation occurs (see Basic Protocol 3, steps 1 and 2).
28. Store confirmed transformants in glycerol stocks (see Basic Protocol 1).

It is recommended to save 2 to 3 replicates.

BASIC PROTOCOL 7

CO-CULTURING FUNGI AND BACTERIA FOR EXTRACTION OF SECONDARY METABOLITES

Fungal SM profiles change when fungi are subjected to different conditions, one being the presence of another organism. There is growing interest in understanding the implications of fungal-bacterial interactions, and activation of BGCs is an important aspect to be explored. In the search for novel SMs, co-culturing is useful for inducing expression of metabolites that otherwise are not expressed. This protocol is adapted from prior work (Stroe et al., 2020) and describes methods used to co-culture fungi and bacteria for the production and extraction of SMs produced exclusively under co-culture conditions. The medium used for strain activation and co-culturing can be modified for different strains and depending on the goal of the experiment. SMs produced under co-culturing conditions on one medium will not necessarily be produced on an alternate medium. The reader is also referred to specific methods of *A. fumigatus*/bacteria co-culture (Zheng, Keller, & Wang, 2015).

Materials

Plate of bacterial strain
BHI liquid medium (see recipe) or other rich medium
Sterile 33% (v/v) glycerol (e.g., Fisher, cat. no. 327255000)
BHI agar plate (see recipe) or other rich medium agar plate
CPG liquid medium (see recipe) or other rich medium
1× phosphate-buffered saline (PBS; e.g., Corning, cat. no. 21-040-CV; optional)
Spore suspension of fungal strain (see Basic Protocol 2)
GMM liquid medium (see recipe) or other minimal medium
GMM agar plate (see recipe)

Round-bottom polystyrene culture tubes (e.g., Falcon, cat. no. 9217F80)
Incubator (e.g., MaxQ 4000, cat. no. SHKE4000), 30° to 37°C and 37°C

Vortex

Cryogenic tubes (e.g., VWR, cat. no. 89004-320)
1.5-ml microcentrifuge tubes (e.g., VWR, cat. no. 111564; optional)
Microcentrifuge (e.g., Eppendorf Centrifuge 5424, cat. no. 05-400-002; optional)
OD reader (e.g., Thermo SPECTRONIC; optional)
250-ml Erlenmeyer flasks
Miracloth (e.g., Merck Millipore, cat. no. 475855)

Additional reagents and equipment for extraction of metabolites (see Basic Protocol 3 and Alternate Protocols 2 to 4)

Creation of bacterial glycerol stocks

1. Using a plate of the bacterial strain, inoculate a single colony into a round-bottom polystyrene culture tube with 7 ml BHI liquid medium or other rich medium.

For human bacterial isolates, BHI is typically used; for other bacteria, CPG or another rich medium that supports growth will work fine. Stroe et al. (2020) use TSB for growing Streptomyces.

2. Incubate culture at the appropriate growth temperature (30° to 37°C) overnight.
3. Vortex grown culture until homogenous and inoculate 1 ml of the bacterial culture into a cryogenic tube with 1 ml sterile 33% glycerol.
4. Vortex tube to ensure a homogenous mixture.
5. Store at -80°C.

Preparation of bacteria

6. Streak out bacteria from a freezer stock (see step 5) onto a BHI agar plate or other rich medium agar plate for isolation of a single colony.
7. Incubate plate at the appropriate temperature (30° to 37°C) for 24 to 48 hr or until colonies form.
8. From the plate, inoculate a single colony into 3 ml CPG liquid medium or other rich medium in a round-bottom polystyrene culture tube.

*For strains such as *Streptomyces* spp. which are spore forming, spores can be collected and used to inoculate a culture instead of the colony method.*

9. Incubate culture at the appropriate growth temperature (30° to 37°C) overnight.

This culture should be started the day before setting up a co-culture experiment (see steps 19a to 25a and 19b to 22b). Specific timing varies by strain. To quantify the cells most accurately based on OD₆₀₀, the cultures should not be overgrown. Usually ~16 hr (no more than 20 hr) works well, but some strains cannot go much past 16 hr.

Washing steps [optional; if inoculating into a medium that differs from the overnight culture (e.g., BHI to GMM)]

10. Transfer three 1-ml aliquots of overnight culture into 1.5-ml microcentrifuge tubes.
11. Centrifuge 5 min at 1500 × g.
12. Pipet off supernatant.
13. Add 1 ml of 1× PBS to each tube.
14. Centrifuge 5 min at 1500 × g.
15. Pipet off supernatant.
16. Repeat steps 13 to 15 for a second wash.
17. Concentrate cells in 1× PBS as desired based on growth and the number of cells needed.
18. Take an OD₆₀₀ reading using an OD reader to quantify bacterial cells.

For some strains, there are published papers with estimates for conversion from OD₆₀₀ reading to cell number. If there is no estimate available and you do not know the conversion for your strain, it is a good idea to create your own OD₆₀₀-to-CFU calibration curve to determine this before proceeding.

Option 1: Co-culturing in liquid medium

- 19a. Inoculate 10⁸ spores of *A. fumigatus* from spore suspension of the fungal strain into 50 ml GMM liquid medium or other minimal medium into a 250-ml Erlenmeyer flask.

- 20a. Incubate at 37°C for ~16 hr.
- The growth temperature should be optimized based on the strain being used.*
- 21a. Use Miracloth to separate grown mycelia from GMM medium.
- 22a. Place collected mycelia into 50 ml fresh GMM liquid medium into a 250-ml Erlenmeyer flask.
- 23a. Add bacteria from the overnight pre-culture from step 9 to the GMM flask with grown mycelia from step 22a.

*The paper that this protocol is adapted from uses 1/20 of an overnight *Streptomyces* culture for inoculation. Typically, for other interaction studies, ratios of 1:100 bacterial to fungal cells work well due to the difference in growth rate. Regardless of the inoculum size, most bacteria will grow rapidly in 12 to 24 hr.*

The medium may need to be modified depending on what will support growth of the bacterium being used. Some bacteria are adaptable and able to grow in minimal medium, whereas others cannot or will take a long time to grow.

- 24a. Incubate at 37°C for 12 hr.
- 25a. Extract metabolites for chemical analysis (see Alternate Protocol 4).

Option 2: Co-culturing on solid medium

- 19b. Inoculate 10³ spores of *A. fumigatus* from spore suspension of the fungal strain onto one side of a GMM agar plate in a 10-µl spot. Incubate at 37°C for ~16 hr.

The growth temperature should be optimized based on the strain being used.

- 20b. In a 10-µl spot 10 mm away from the fungal spot, inoculate bacteria from step 9 diluted to an OD of 0.1.
- 21b. Incubate at 37°C for 48 hr.
- 22b. Follow Basic Protocol 3 or Alternate Protocol 2 or 3 for extracting metabolites from solid medium for chemical analysis.

REAGENTS AND SOLUTIONS

BHI liquid medium and agar plates

37 g Brain Heart Infusion (BHI) broth (e.g., Millipore Sigma, cat. no. 53283; 3.7% w/v)

ddH₂O to 1 L

Autoclave

Store liquid medium ≤5 years at room temperature

For plates, add 16 g agar (1.6% w/v) dissolved in ddH₂O before autoclaving

Pour plates

Store plates in sleeve ≤2 months at 4°C or until dry

When pouring plates for SM experiments, it is recommended to use motorized pipets for consistency. This is not necessary for activating strains.

Fresher plates are always recommended for experiments.

CPG liquid medium

700 ml ddH₂O

1 g casein acid hydrolysate (e.g., Millipore Sigma, cat. no. 91079-40-2; 0.1% w/v)

1 g yeast extract (e.g., Millipore Sigma, cat. no. 8013-01-2; 0.1% w/v)

10 g peptone (e.g., Fisher, cat. no. 211677; 1% w/v)

10 g glucose (dextrose) (e.g., Fisher, cat. no. CAS 50-99-7; 1% w/v)

ddH₂O to 1 L
Autoclave
Store liquid medium \leq 5 years at room temperature

Czapek-Dox Broth

700 ml ddH₂O
30 g sucrose (e.g., Fisher, cat. no. 15503022; 3% w/v)
3 g sodium nitrate (0.3% w/v)
1 g dipotassium phosphate (e.g., Sigma-Aldrich, cat. no. 340448; 0.1% w/v)
0.5 g MgSO₄ (e.g., Sigma-Aldrich, cat. no. M7506-500G; 0.05% w/v)
0.5 g potassium chloride (e.g., Sigma-Aldrich, cat. no. P3911; 0.05% w/v)
10 mg ferrous sulfate (e.g., Sigma-Aldrich, cat. no. 1270355; 0.001% w/v)
Adjust pH to 7.3 with NaOH
ddH₂O to 1 L
Autoclave
Store \leq 5 years at room temperature
Add in the order listed. Heat if needed to help dissolve the compounds.

Glucose Minimal Medium (GMM) liquid medium and agar plates

700 ml ddH₂O
50 ml 20 \times nitrate salts (see recipe)
1 ml trace elements (see recipe)
10 g glucose (dextrose; e.g., Fisher, cat. no. CAS 50-99-7; 1% w/v; substitute xylose if making XMM)
Add any supplements needed for auxotrophic mutants
For GMM used in transformations, add 0.5 g yeast extract (e.g., VWR, cat. no. 97064-368; 0.05% w/v)
Adjust pH to 6.5 with NaOH (1 to 2 M NaOH typically works best)
ddH₂O to 1 L
Autoclave
Store liquid medium at room temperature in clear bottle until signs of contamination
For plates, add 16 g agar dissolved in ddH₂O (1.6% w/v) before autoclaving
Autoclave
Add any selective agents
Combine with liquid medium
Pour plates
Store plates in sleeve \leq 2 months at 4°C or until dry
When pouring plates for SM experiments, it is recommended to use motorized pipets for consistency. This is not necessary for activating strains.
Fresher plates are always recommended for experiments.
GMM is sometimes referred to as Aspergillus Minimal Medium (AMM), but the recipe may vary slightly depending on the variation that is being made. Xylose Minimal Medium (XMM) is identical but uses xylose in replacement of glucose.

LB liquid medium and agar plates

700 ml ddH₂O
5 g yeast extract (e.g., VWR, cat. no. 97064-368; 0.5% w/v)
10 g peptone (e.g., Fisher, cat. no. 211677; 1% w/v)
10 g NaCl (e.g., Fisher, cat. no. BP358-1; 1% w/v)
Adjust pH to 7.0 with NaOH
ddH₂O to 1 L
Autoclave

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Store liquid medium at room temperature in clear bottle until signs of contamination
For plates, add 16 g agar (1.6% w/v) dissolved in ddH₂O before autoclaving
Autoclave
Pour plates
Store plates in sleeve \leq 2 months at 4°C or until dry
When pouring plates for SM experiments, it is recommended to use motorized pipets for consistency. This is not necessary for activating strains.
Fresher plates are always recommended for experiments.

Liquid Minimal Medium (LMM)

700 ml ddH₂O
10 g glucose (dextrose; e.g., Fisher, cat. no. CAS 50-99-7; 1% w/v)
50 ml 20× nitrate salts (see recipe)
1 ml trace elements (see recipe)
For LMM used in transformations, add 0.5 g yeast extract (e.g., VWR, cat. no. 97064-368; 0.05% w/v)
Add any supplements
Adjust pH to 6.5 with NaOH (1 to 2 M NaOH typically works best)
ddH₂O to 1 L
Autoclave
After autoclaving, add any selective agents
Store \leq 5 years at room temperature with foil over lid

Malt extract agar

700 ml ddH₂O
20 g malt extract (e.g., VWR, cat. no. IC15531590; 2% w/v)
20 g glucose (e.g., Fisher, cat. no. CAS 50-99-7; 2% w/v)
1 g peptone (e.g., Fisher, cat. no. 211677; 0.1% w/v)
10 mg ZnSO₄·7H₂O (e.g., Fisher Scientific, cat. no. AC424605000; 0.001% w/v)
5 mg CuSO₄·5H₂O (e.g., Fisher Scientific, cat. no. BP346-500; 0.0005% w/v)
Adjust pH to 4.7 with NaOH (1 to 2 M NaOH typically works best)
Add 16 g agar (1.6% w/v)
ddH₂O to 1 L
Autoclave
Pour plates
Store plates in sleeve \leq 2 months at 4°C or until dry

It can be difficult to fully dissolve the compounds. Bringing the solution to a boil for 1 min on a hot plate while stirring can help. Alternatively, dissolve the malt extract in 500 ml ddH₂O and the rest of the compounds in 500 ml ddH₂O. Autoclave separately and then mix.

When pouring plates for SM experiments, it is recommended to use motorized pipets for consistency. This is not necessary for activating strains.

Fresher plates are always recommended for experiments.

Mycelium wash

500 ml ddH₂O
147.9 g MgSO₄ (e.g., Sigma-Aldrich, cat. no. M7506-500G; 14.7% w/v or 0.6 M final)
ddH₂O to 1 L
Autoclave
Store \leq 5 years at 4°C

Nitrate salts, 20×

700 ml ddH₂O
120 g sodium nitrate (e.g., Fisher, cat. no. S343-500; 12% w/v)
10.4 g KCl (e.g., Fisher, cat. no. BP366-500; 1.04% w/v)
10.4 g MgSO₄ (e.g., Sigma-Aldrich, cat. no. M7506-500G; 1.04% w/v)
30.4 g KH₂PO₄ (e.g., Fisher, cat. no. BP363-1; 3.04% w/v)
ddH₂O to 1 L
Autoclave
Store ≤5 years at room temperature.

Oatmeal medium

700 ml ddH₂O
16 g old-fashioned oatmeal (1.6% w/v)
Any supplements needed for auxotrophic mutants
Homogenize in blender for 5 min
ddH₂O to 1 L
Autoclave
Store ≤5 years at room temperature with foil over lid
This is an alternative commonly used medium in metabolite extraction experiments.

Osmotic medium

1.2 M (147.9 g) MgSO₄ (e.g., Sigma-Aldrich, cat. no. M7506-500G)
10 mM sodium phosphate (e.g., Sigma-Aldrich, cat. no. 342483)
ddH₂O to 500 ml or 1 L
Adjust pH to 5.8 with 1 M Na₂HPO₄
Filter sterilize
Store ≤5 years at room temperature

PEG solution

60% PEG 4000 or 6000 (e.g., Calbiochem, cat. no. 528877)
50 mM or 0.234% (2.35 g) CaCl₂ (e.g., Dot Scientific, cat. no. DSC20010-1000)
50 mM Tris-HCl, pH 7.5 (see recipe for 10 mM Tris buffer, pH 8)
ddH₂O to 1 L
Autoclave
Store ≤(maximum storage duration?) at room temperature

Potato Dextrose Broth (PDB) and Agar (PDA)

24 g dehydrated PDB (e.g., BD, cat. no. 213400; 2.4% w/v)
ddH₂O to 1 L
Autoclave
Store PDB at room temperature in clear bottle until signs of contamination
For plates, add 16 g agar dissolved in ddH₂O (1.6% w/v) before autoclaving
Pour plates
Store PDA plates in sleeve ≤2 months at 4°C or until dry
When pouring plates for SM experiments, it is recommended to use motorized pipets for consistency. This is not necessary for activating strains.
Fresher plates are always recommended for experiments.

Stabilized Minimal Medium (SMM) agar plates

500 ml ddH₂O
10 g glucose (dextrose; e.g., Fisher, cat. no. CAS 50-99-7; 1% w/v)
50 ml 20× nitrate salts (see recipe)
1 ml trace elements (see recipe)
218.6 g sorbitol (e.g., Sigma, cat. no. S6021-1KG; 1.2 M w/v)

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Add any supplements if needed
For SMM used before transformation, add 1 g yeast extract (e.g., VWR, cat. no. 97064-368; 0.1% w/v)
Adjust pH to 6.5 with NaOH
Add 16 g agar as bottom agar (1.6% w/v)
Add 7.5 g agar as top agar (0.75% w/v)
ddH₂O to 1 L
Autoclave
Store \leq 5 years at room temperature

STC buffer

700 ml ddH₂O
1.2 M (218.6 g) sorbitol (e.g., Sigma, cat. no. S6021-1KG)
10 mM (0.47 g) CaCl₂ (e.g., VWR, cat. no. 97062-590)
10 mM Tris-HCl, pH 7.5 (see recipe for 10 mM Tris buffer, pH 8)
ddH₂O to 1 L
Autoclave
Store \leq 5 years at 4°C

Trace elements

50 ml ddH₂O
2.20 g ZnSO₄·7H₂O (e.g., Fisher, cat. no. Z68-500; 0.22% w/v)
1.10 g H₃BO₃ (e.g., Fisher, cat. no. A74-500; 0.11% w/v)
0.50 g MnCl₂·4H₂O (e.g., Fisher, cat. no. BP214-500; 0.05% w/v)
0.16 g FeSO₄·7H₂O (e.g., Sigma-Aldrich, cat. no. 026-003-01-4; 0.016% w/v)
0.16 g CoCl₂·6H₂O (e.g., Fisher, cat. no. AC423570050; 0.016% w/v)
0.16 g CuSO₄·5H₂O (e.g., Fisher, cat. no. 18-601-914; 0.016% w/v)
0.11 g (NH₄)₆Mo₇O₂₄·4H₂O (e.g., Sigma-Aldrich, cat. no. 12054-85-2; 0.011% w/v)
5.00 g ethylenediamine tetraacetic acid, disodium salt dihydrate (e.g., Fisher, cat. no. S311-3; 0.5% w/v)
ddH₂O to 100 ml
Store \leq 5 years at room temperature

Dissolve each item in this exact order, making sure each is dissolved. You may need to add KOH to help fully dissolve everything.

Trapping buffer

600 ml ddH₂O
0.6 M (109.3 g) sorbitol (e.g., Sigma, cat. no. S6021-1KG)
0.1 M Tris-HCl, pH 7 (see recipe for 10 mM Tris buffer, pH 8)
ddH₂O to 1 L
Autoclave
Store \leq 5 years at 4°C

Tris buffer (pH 8), 10 mM

700 ml ddH₂O
1.22 g Tris (e.g., VWR, cat. no. 0497-5KG)
Adjust pH to 8 with HCl
ddH₂O to 1 L
Store \leq 5 years at room temperature

If making at an alternative pH or molarity, adjust as necessary.

To make the preparation easier, make a stock solution of 1 M Tris buffer and dilute to 10 mM when needed.

Tween water

0.01% Tween 80 (e.g., MP Biomedicals, cat. no. 103170)

ddH₂O to 1 L

Autoclave

Store ≤5 years at room temperature

To make the preparation easier, make a stock solution of 10% Tween 80 in ddH₂O and add 1 ml to 1000 ml ddH₂O when more Tween water is needed.

YPAD medium, 2×

700 ml ddH₂O

20 g yeast extract (e.g., VWR, cat. no. 97064-368; 2% w/v)

40 g peptone (e.g., BD, cat. no. 211677; 4% w/v)

40 g glucose (dextrose) (e.g., Fisher, cat. no. CAS 50-99-7; 4% w/v)

80 mg adenine hemisulfate (e.g., Sigma-Aldrich, cat. no. A9126; 0.008% w/v)

ddH₂O to 1 L

Autoclave

Store ≤5 years at room temperature

If making solid YPAD, add agar and store at 4°C.

COMMENTARY

Background Information

Chemists were at the forefront of early research into fungal SMs, with a strong emphasis being on function and characterization (Raistrick, 1950). In recent decades, with the advent of next-generation sequencing technologies and the subsequent bioinformatics boom, significant efforts have been able to link metabolites to specific BGCs, with high rates of success in *A. fumigatus* (Raffa & Keller, 2019; Wang, Yu, et al., 2021). The rise of genome-mining software such as SMURF and ANTISMASH has streamlined the process for identifying putative BGCs in plants, bacteria, and fungi (Blin et al., 2019; Khaldi et al., 2010). However, there exists a major bottleneck in SM research: most BGCs are silent under normal laboratory conditions, as they are tightly controlled by global and cluster-specific regulators (Keller, 2019).

Over the years, the fungal SM research community has developed two broad approaches to “turning on” silent BGCs. The first strategy, which broadly involves altering the physical growth conditions, has been dubbed “OSMAC” (one strain, many compounds) (Bode, Bethe, Höfs, & Zeeck, 2002; Schiewe & Zeeck, 1999). This typically involves altering environmental growth conditions such as medium, growth temperature, light, and pH (Bayram et al., 2008; Bills et al., 2008; Frisvad, 2012). For an excellent literature review on the numerous ways that this approach has been leveraged, see Pan, Bai, Chen, Zhang, & Wang (2019). The OSMAC strat-

egy has become less effective in recent years due to the high rediscovery rate of known SMs. Co-culturing and use of nontraditional species are potential exceptions to this, as these species have been historically understudied (Stroe et al., 2020; Krause et al., 2018; Nützmann et al., 2011). With the decreasing cost of sequencing fungi, a complementary approach involving both chemistry and genomics (metabologenomics) and use of heterologous expression systems can place BGCs into gene cluster families to reduce rediscovery and provide a more rational means for new compound breakthroughs (Robey et al., 2020).

The second strategy involves mining fungal genomes for BGCs and utilizing genetic engineering to induce specific clusters. This allows for heterologous expression of entire clusters in model systems such as *A. nidulans* or homologous editing of global/local transcription factors that might activate silent clusters (Nielsen et al., 2013; Oakley et al., 1987; Raffa et al., 2021; Tilburn et al., 1995; Zhang et al., 2020). Early transformation technologies were pioneered in the model filamentous fungus *A. nidulans*. Today, *A. nidulans* still serves as the gold standard for heterologously expressed BGCs (Caesar et al., 2020).

There are three major disadvantages to this technology. The developed genetic tools in filamentous fungi have been mostly limited to select Ascomycota species that are culturable and capable of conidiation under laboratory conditions. This includes many of the basic protocols detailed in this article

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(e.g., Basic Protocols 4 and 6). Additionally, the difficulty of the methods themselves hinders labs that are not familiar with the techniques from successfully implementing them, something we hope to address in part with this article. Lastly, traditional transformation methods in filamentous fungi rely on the HR pathway to replace the endogenous segment with the introduced DNA construct. With some notable exceptions (i.e., the budding yeast subphylum), most species in the fungal kingdom favor NHEJ over HR, resulting in very low frequencies of gene replacement. This has led to the increased use of ΔkuA and ΔkuB strains (exact gene names may vary between species), which, when mutated, forces the fungus to only utilize HR. Ku70 and Ku80 are two conserved core subunits in the heterodimeric Ku protein complex that mediates the NHEJ pathway. Mutation of either Ku70 or Ku80 protein can increase the frequency of gene replacement 10- to 30-fold, depending on the species (Krappmann, 2007).

Within the last 6 years, there has been an exciting push toward creating CRISPR-Cas9 systems that can genetically engineer filamentous fungi (Nødvig, Nielsen, Kogle, & Mortensen, 2015; Song et al., 2019), including *A. fumigatus* (van Rhijn et al., 2020). As of 2019, CRISPR-Cas9 genome-editing systems had been developed in over 40 filamentous fungi and oomycetes, as reviewed in Schuster & Kahmann (2019). One of the recent CRISPR breakthroughs for the fungal natural products field came from the development of CRISPR-mediated transcriptional activation (CRISPRa). Using a nuclease-deactivated CRISPR/Cas complex, researchers were able to repurpose it as an artificial transcription factor capable of modulating gene expression. This allows for the activation of multiple genes via multiple guide RNAs, which was utilized by the original authors to activate a silent non-ribosomal peptide synthetase (NRPS) cluster in *A. nidulans* (Roux et al., 2020). There is no doubt that CRISPR-based technologies will begin playing a larger role in the future of the fungal natural products field. This will be vital for reducing the time that it takes to activate silent clusters, in addition to enabling the manipulation of nontraditional organisms.

Critical Parameters

Basic Protocol 3

Whenever running a secondary metabolism extraction, the utmost care must be taken with the growth conditions. As an example, if one

follows a previous publication perfectly but decides to grow the strain at room temperature instead of 37°C as was listed in the publication, there is a chance that the SM will not be produced in the same quantity. Take detailed laboratory notes and carefully plan out your experiments before conducting them.

Basic Protocol 4

During the DNA extraction, the critical step is incubating until there is a large mycelial mat on the dish. This can be tricky to get right, as in our lab's experience, the optimal time to grow a strain may vary from experiment to experiment. If it appears that some of your strains are ready for DNA extraction but that other petri dishes need to be grown more, place the ready plates in a 4°C refrigerator to slow down growth. Remember, if a plate has already made a large number of spores, you must start this step over for that sample.

Basic Protocol 6

There are two tricky steps in the transformation protocol that require observations under a microscope before moving on. This may be daunting to those running the protocol for the first time, as they may be unsure of what constitutes "perfect" versus "under/overgrown." When creating germlings, you should see spores that have small bumps protruding from them. Long hyphal branches are what you want to avoid. When creating the protoplasts, look for a ball inside of a ball under the microscope (a vacuole inside the cell). Protoplasts will appear larger than spores, making them easier to distinguish. It is impossible to have 100% protoplasts, but having a majority is desirable.

Troubleshooting

Please see Table 2 for a troubleshooting guide.

Understanding Results

Extracting secondary metabolites

To verify that your extraction (Basic Protocol 3 and Alternate Protocols 2 to 4) worked, analytical chemistry methods need to be employed. Thin-layer chromatography and UV-based methods are relatively fast and great if you know the compound for which you are looking. When a higher resolution of data is needed, MS, crystallography, and NMR can be excellent tools. Although MS (i.e., LCMS) can be run on crude samples, your compound of interest must be purified before running on NMR or crystallography. As stated earlier,

Table 2 Troubleshooting Guide for Common Issues in Fungal Secondary Metabolism Lab Work

Problem	Possible cause	Solution
My metabolite of interest is not showing up during chemical analysis	The gene cluster that produces the metabolite is silent under the growth conditions tested	<ol style="list-style-type: none">1. Use the OSMAC strategy and change the growth variables.2. Make sure that you are using the proper organic solvent during the extraction. If unsure what to use, ethyl acetate and chloroform are the best for picking up the widest diversity of polar and non-polar compounds.3. If the BGC has an internal transcription factor, transform the strain to overexpress it.4. You might need to utilize heterologous expression to activate the silent gene cluster (see Basic Protocols 4 to 6).
After running the transformation, none of the replicates tested via PCR, Southern blotting, or sequencing showed the correct insertion	The rate of gene recombination in some strains can be as low as 2%, and thus, recombination can be very rare (Zhang et al., 2011)	<ol style="list-style-type: none">1. You may not have tested enough replicates. When using non-ΔKu70/Ku80 backgrounds, it is recommended to test a minimum of 50 strains. It is common for the vast majority to be false positives, even when grown on selective medium. Test more of the isolates from the transformation.2. Consider using a ΔKu70/Ku80 background if available for your strain. Make sure that the pleiotropic impacts of doing so will not hinder any conclusions being drawn from the studies.3. Sometimes the DNA construct itself needs to be remade, perhaps with different primer sets and overhang regions.
The metabolite extraction looks very cloudy	Agarose or fungal tissue is inside of the sample and needs to be removed before running on HPLC/LCMS-MS or similar	Filter the solution multiple times if needed. Alternatively, you can centrifuge the sample to pellet the insoluble materials. It is important to have a very clean sample to prevent clogging the instrumentation used in chemical analysis.
My strain did not grow properly or sporulate on GMM	The medium is not optimal for your species/strain	<ol style="list-style-type: none">1. Many species will never sporulate under laboratory conditions. Although this can make molecular biology techniques that rely on spores incredibly difficult, if not impossible, to conduct, metabolic extractions from the mycelium still might yield interesting results.2. Ensure that you have added the necessary supplements if relevant. Additionally, look to the literature to see in what other groups commonly grow your strains/species. Although commonly used for a wide array of fungi, GMM can be substituted with an alternative medium in all protocols detailed.

(Continued)

Table 2 Troubleshooting Guide for Common Issues in Fungal Secondary Metabolism Lab Work, *continued*

Problem	Possible cause	Solution
I am having frequent contamination issues	You need to take appropriate measures to ensure sterility	<p>It is incredibly easy to have accidental contamination in fungal laboratories due to the increased number of spores present in the air. Ensure that you are following these sterile technique rules of thumb:</p> <ol style="list-style-type: none"> 1. Anything that touches a live culture should only be opened in a sterile biosafety cabinet. 2. Use filter tips when doing any work that involves live cultures. 3. Avoid using a Bunsen burner for sterile steps. Although commonly used in non-sporulating yeast and bacteria labs, it often will not properly prevent contamination in filamentous fungi labs. 4. In our experience, some researchers have a natural skin microbiota that makes them more prone to causing contamination during their experiments (despite taking other precautions). Consider double gloving if worried that this may be the case for yourself. 5. The only way to ensure that a biosafety cabinet is sterile between strains/replicates in an experiment is to turn on a UV light for 15 min between samples. Although this is not always necessary, it is highly recommended when creating or using glycerol stocks, especially if said strain has no selectable marker.
My point inoculations are not growing as a single circle in the center of the plate	You are moving the plate before the spore suspension can dry or are pipetting the liquid out too forcefully	<p>To ensure the tightest possible point inoculation, pipet the spore suspension very slowly and avoid pushing to the second stop. Allow the spore suspension to completely dry before moving. If in a biosafety cabinet, this can be quickly done by leaving the lid off the plate for 10 to 20 min. Otherwise, let the plate sit at room temperature for several hours until dry before moving.</p>
Fungus or bacterium is not growing well Bacterium is not growing in minimal medium	Growth of one is overpowering the other The medium is missing essential nutrients	<p>Try to modify the ratio of the initial inoculum to use more cells of the one that is not growing well.</p> <p>The minimal medium sufficient to support fungal growth will not always be effective for bacteria. Try supplementing the medium listed here with any missing components that are required for your bacterium to grow.</p>

there are great reviews (Bucar et al., 2013; Raja et al., 2017; Sticher, 2008) with more details on compound identification.

Verifying that the transformation was successful

After you have completed the transformation protocol and have small colonies growing on the selective medium, transfer the colonies to their own isolate plates (Basic Protocol 6). This is best if done before conidiation has occurred to ensure that you are transferring only from one colony. You should transfer a minimum of 20 isolates, but more is better. Extract the genomic DNA from the isolates for PCR verification with screening primers. For the isolates that are confirmed with PCR, double-check that the insertion recombined into the correct location using Southern blotting or DNA sequencing. It is recommended to save several replicates of successful transformations as glycerol stocks for future use.

Time Considerations

Making (Basic Protocol 1 and Alternate Protocol 1) and activating glycerol stocks (Basic Protocols 2 and 7 and Support Protocol) can be done in several hours, followed by several days of allowing the fungus to grow, and are very routine processes. The only necessary preparation is ensuring that you have made the proper medium for plating out the fungus. Similarly, extracting metabolites from fungal cultures (Basic Protocol 3 and Alternate Protocols 2 to 4) is a relatively routine and simple task. The most challenging step is determining which organic solvent is best for your compound of interest. Depending on how long the culture is sonicated or mixed, the process can take anywhere from a single hour to overnight.

Genetic transformations (Basic Protocols 4 to 6 and Alternate Protocol 5) are more time consuming. From start to finish, if everything works well, the process can be completed in full within 3 to 4 weeks. The transformation itself takes ~2 days. Verification of successful transformants takes ~1 week. The rest of the time is taken up waiting for the fungus to grow.

Overall, in the best-case scenario, where your SM of interest is expressed under common growth conditions, one can obtain a crude sample within 2 to 3 weeks. Most of that time is taken up by allowing the fungus to grow. In the worst-case scenario, you may never get your BGC to be turned on despite months (if not years, if you are not careful) of effort. The OSMAC approach can quickly become over-

whelming with the pairwise combinations of conditions that can be changed. Months can be spent trying various media, temperatures, plating methods, and so on. When trying to genetically activate BGC expression, internal transcription factors may not always regulate the BGC adjacent to it (Wang, Drott, et al., 2021). Additionally, attempts to heterologously express entire clusters in model organisms are not always fruitful.

It is important to bear the above in mind. Knowing when your time would be better used pursuing alternative avenues is crucial to researching natural products. There are numerous cases of researchers pursuing a specific BGC but never succeeding in turning on its transcription despite months of work. SMs are highly attuned compounds that improve the fitness of the organism in which they evolve, and there can be significant variation even within the same species (Drott et al., 2020). It may go without saying that the natural products community only understands a small fraction of the complex regulatory networks that control natural products' underlying expression.

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Grant Nickles: Conceptualization, Data curation, Funding acquisition, Visualization, Writing-original draft, Writing-review and editing; **Isabelle Ludwikoski:** Writing-original draft, Writing-review and editing; **Jin Woo Bok:** Writing-review and editing; **Nancy P. Keller:** Funding acquisition, Supervision, Writing-review and editing.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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Data Availability Statement

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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- ### Internet Resources
- <https://macro.lsu.edu/howto/solvents/Polarity%20index.htm>
List of polarity indexes of commonly used solvents created by Honeywell - Burdick & Jackson.
- <https://www.mrc-lmb.cam.ac.uk/ms/methods/phenol.html>
Phenol/chloroform/isoamyl alcohol preparation method listed on the MRC Laboratory of Molecular Biology (Cambridge, UK) website, with added details on phenol preparation, notes on buying with stabilizers, and extra safety warnings.
- <https://www.cdc.gov/niosh/idlh/108952.html>
CDC safety sheet on phenol, citing the minimum amount needed to be a lethal dose.
- http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html
Resource published by Premier Biosoft that very thoroughly covers good practices in primer design; following these rules will vastly improve the chances that your primers work on the first try.
- <http://www.fgsc.net/aspergillus/protocols/mediaforaspergillus.pdf>
*List of commonly used *Aspergillus* media.*
- https://www.fpl.fs.fed.us/documents/pdf2004/fpl_2004_nakasone001.pdf
Details the preservation and distribution of fungal cultures in great detail.