Mar 24, 2025

Fungal Isolation and Cell DNA (c-DNA) Extraction from Soil Samples

DOI
https://dx.doi.org/10.17504/protocols.io.3byl4w1krvo5/v1

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Protocol Citation: Nuttapon Pombubpa, Ariya Chindamporn, Nattapol Kraisitudomsook, Neil Andrew Robert Gow, Syahriar Nur Maulana Malik Ibrahim, Fikran Aranda Fahrudin 2025. Fungal Isolation and Cell DNA (c-DNA) Extraction from Soil Samples. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl4w1krvo5/v1

Protocol status: In development
We are still developing and optimizing this protocol

Created: January 29, 2025

Last Modified: March 24, 2025

Protocol Integer ID: 119317

Keywords: Fungal isolation, WHO FPPL, Fungal genome extraction, Fungal identification, Soil fungal, accurate responses to fungal threat, extracting fungal genomic dna, resistant fungi, fungal genomic dna, fungal pathogen, antifungal resistance, fungal threat, several pathogenic fungi, antifungal resistance to major drug class, fungi, fungal isolation, agricultural biosecurity, strategies against fungal infection, fungal infection, including fungi, including fusarium group, fusarium group, leveraging genomic technology, specific resistance gene, diverse microorganism, comprehensive genome database, genomic technology, agricultural soil, dna extraction, collecting soil sample, prevalent in thailand, eumycetoma agent, soil samples soil, genomic dna, soil sample, extraction from soil samples soil, significant human infection, caused significant human infection, network of mycologist, soil, humans through infection

Funders Acknowledgements:
FAILSAFE Awarded projects - Seed Grant
Grant ID: FAILSAFE FR1-23

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Abstract

Soil harbors diverse microorganisms, including fungi that play crucial environmental roles but can also harm plants, animals, and humans through infections or spore exposure. In 2022, the WHO released several pathogenic fungi, including Fusarium group, Mucorales and Eumycetoma agents, which are prevalent in Thailand's agricultural soils and have caused significant human infections. Despite this, the genetic mechanisms behind their infections and antifungal resistance remain poorly understood, particularly in Southeast Asia. Antifungal resistance to major drug classes, such as azoles, echinocandins, and polyenes, is increasing, driven by specific resistance genes. To address these challenges, we propose establishing a Southeast Asian (SEA) network of mycologists to study fungal pathogens, focusing on collecting soil samples, extracting fungal genomic DNA, and creating a comprehensive genome database. This initiative will improve detection and management of drug-resistant fungi, enhance clinical treatments, and strengthen agricultural biosecurity. By leveraging genomic technologies and fostering international collaboration, the project aims to develop targeted strategies against fungal infections and resistance, benefiting public health, food security, and sustainable agriculture across SEA. Establishing a fungal biobank and expert network will support long-term efforts to combat antifungal resistance, providing critical resources for swift and accurate responses to fungal threats.

Attachments

PDF Hand Book_AllPrep_Fu... 256KB

PDF 1998 Barnet & Hunter... 21.5MB

Materials

Molecular process

- AllPrep Fungal DNA Kit (QIAGEN)
- Agarose and Electrophoresis Set
- TBE Buffer
- DNA Ladder
- PCR master mix with Mg^{2+}, dNTPs, and Taq DNA polymerase
- ITS1 and ITS4 primer set

Isolation process

- Sterile Water, for dilution (10^{-3} to 10^{-6})
- Spread Rod, for cultivation
- Petri Dish
- Glass Rod, for spread plate cultivation
- V-rod glass or steel, for slide culture
- Object and Cover Glasses

Culture Media:

- Potato Dextrose Agar (PDA)
- Potato Dextrose Broth (PDB)
- Malt Extract Agar (MEA)
- Antibacteria (Tetracyclin 40 mg/L)

Fungal Isolation

1 **Sample Preparation**

1.1 Weigh 1 g of soil sample.

1.2 Perform serial dilutions of soil using sterile water (10^{-3} to 10^{-6} dilutions with 9 mL sterile water).

2 **Cultivation Using Spread Plate Method**

2.1 Spread 100 µL of each dilution onto culture media (PDA and MEA) that contains antibacteria (tetracyclin 40 mg/L).

2.2 Incubate plates at room temperature (25-30 ºC) for 5–7 days until fungal colonies are visible.

3 **Colony Purification**

3.1 Transfer fungal hyphae from mixed colonies to fresh PDA plates for purification.

3.2 Incubate at room temperature (25-30 ºC) for 5–7 days to obtain pure colonies.
Note: Repeat this step until a single culture is obtained.

4 **Fungal Identification**

4.1 ***Colony morphology and microscopic documentation***

The colony features based on:
- **Color** (top and reverse)
- **Margin** (smooth/entire, filamentous, lobed, or irregular)
- **Elevation** (flat, raised, umbonate (with a central bump), or wrinkled)
- **Texture** (powdery, velvety, cottony, granular, glabrous, or creamy)
- **Zonation** (presence or not)
- **Radial furrow** (presence or not)
- **Exudate drop** (presence or not)

The microscopic can be prepared by slide culture technique
Prepare slide cultures to observe substrate and aerial hyphal structures. Adding Lactophenol-Cotton Blue stain to the growth colony is recommended for better visualization.
Characterize the cell structure based on:

- **Hyphae** (septate or aseptate)
- **Spore - Asexual** (consisting of macroconidium, microconidium, chlamydospore, arthrospore, blastospore, or sporangiospore)
- **Spore - Sexual** (type of ascospore, basidiospore, or zygospore)
- **Sporangiophore** (consisting of aporangium, columella, or hyphae-stalk)
- **Conidiophore** (type of simple, complex, acrotheca, cladosporium or phialophora)

*Read the technique of slide culture here: https://sea-armi.github.io/research/SlideCulture

4.2 *Fungal identity confirmation*

Compare the colony and slide culture results with fungal book determination.
Reference:

- Barnett & Hunter, 1998. Illustrated Genera of Imperfect Fungi, Fourth Edition
- SEA-ARMi, 2025. Filamentous WHO FPPL target: Mucorales, Eumycetoma causative agents, and *Fusarium* group

**Read* the Barnett & Hunter, 1998. Illustrated Genera of Imperfect Fungi, Fourth Edition at the attached file
**Read* SEA-ARMi, 2025. targeted filamentous WHO-FPPL here: https://sea-armi.github.io/research/TargetedWHO

5 ***Molecular identification***

For molecular identification, single fungal colony DNA extraction follows **Step 7 (cDNA extraction)**.
The fungal genome is amplified using the Internal Transcribed Spacer (ITS) region, with the following primers:

- ITS1 forward primer: 5' TCCGTAGGTGAACCTGCGG 3'
- ITS4 reverse primer: 5' TCCTCCGCTTATTGATATGC 3'

These primers are used to amplify conserved regions of fungal DNA (White et al., 1990).

PCR Mixture **(25 µL total volume)**:

- **12.5 µL** PCR master mix with Taq DNA polymerase
- **0.5 µL** forward primer
- **0.5 µL** reverse primer
- **12 µL** solution (DNA template diluted with molecular-grade water, ensuring a final DNA concentration of 100 ng)

The negative control is PCR mixture without DNA template and the positive control is identified fungal.

Citation

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J. (1990). Amplification and direct sequencing PCR protocols: a guide to methods and applications.

https://www.researchgate.net/publication/223397588_White_T_J_T_D_Bruns_S_B_Lee_a
LINK

5.1 The PCR conditions for amplifying ITS were as follows:
Initial denaturation step at **95°C for 5 minutes**
25 *cycles* of
- **1 minute at 95°C**
- **25 seconds at 56°C**
- **30 seconds at 72°C**

with a *final extension* at **72°C for 5 minutes**
Holding the PCR product at **4°C.**

5.2 The PCR product is analyzed using 1.5% agarose gel electrophoresis, with a 1 Kb DNA ladder as a size marker. Electrophoresis is performed at 100V for 22 minutes in 1× TBE buffer.

5.3 High-quality PCR products will be sequenced using Sanger sequencing, and the resulting sequence files will be identified using BLAST (NCBI database).

6 The pure identified colony can be used for the cDNA extraction.

cDNA Extraction

7 Inoculate the pure fungal colony to PDB and incubate for 5 days to get enough hyphae at room temperature (25-30 ºC) with 100 rpm.

8 Use the **AllPrep Fungal DNA Kit** to extract DNA from the cell hyphae.

8.1 Prepare fungal cell samples and resuspend in Solution HC.

8.2 Perform cell lysis with vortexing.

8.3 Load lysate into MB Spin Columns.

8.4 Wash columns with Solutions RA and RW.

8.5 Elute DNA with Solution EB.

9 **DNA Qualification and Quantification**

9.1 Perform agarose gel electrophoresis to verify DNA quality and purity.

9.2 Use a Nanodrop spectrophotometer to quantify DNA concentration and purity (at least 10 ng/µL).

10 **Submission of Isolated DNA**

10.1 Send the purified fungal DNA to the **FAILSAFE Project Team, SEA-ARMi at Chulalongkorn University** for further analysis.

Citations

Step 5

White, T. J., Bruns, T., Lee, S. J. W. T., & Taylor, J.. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics
https://www.researchgate.net/publication/223397588_White_T_J_T_D_Bruns_S_B_Lee_and_J_W_Taylor_Amplification_and_direct_sequencin