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Isolation of high molecular weight genomic DNA from *Entomophthora muscae*

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protocol .


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Obtaining quality genomic material from Entomophthoralean fungi has proven extremely difficult. This protocol describes a method that has been successfully used to obtain high molecular weight genomic DNA from the obligate fungal pathogen *Entomophthora muscae* for long-read sequencing and genome assembly. The protocol details quality control and quantification steps recommended before proceeding to downstream applications, such as sequencing.

Carolyn Elya 2022. Isolation of high molecular weight genomic DNA from Entomophthora muscae. **protocols.io** https://protocols.io/view/isolation-of-high-molecular-weight-genomic-dna-fro-b6kercte

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Fungal culture

1 Fungal culture can be established from glycerol freezer stock or from a sporulating cadaver following methods outlined in Hajek et al, 2012. Cultures are grown in supplemented Graces Insect Medium **ThermoFisher Scientific Catalog #11605094** with added 5%

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Fetal Bovine Serum **ThermoFisher**

FBS (e.g., **Scientific Catalog #10437010**), which will henceforth be referred to as **Grace's + 5% FBS**.

Hajek AE, Papierok B, Eilenberg J.. Chapter IX - Methods for study of the Entomophthorales. Lacey LA, editor. Manual of Techniques in Invertebrate Pathology (Second Edition).
http://doi:10.1016/B978-0-12-386899-2.00009-9

15m

1.1 **From -80C glycerol stock**

Transfer **20 mL** of **Grace's + 5% FBS** to Falcon 25cm^2 flask **Corning Catalog #353014** and allow to come to room temperature.

Retrieve one **1.5 mL** - **2 mL** aliquot from **-80 °C** and rapidly thaw entire aliquot at **37 °C** .

Transfer entire contents to prepare tissue culture flask.

2d 17h

1.2 **From sporulating cadaver**

Collect conidia via ascending method per Hajek et al, 2012. Briefly, using aseptic technique:

-Adhere cadaver via ventral abdomen to the top of an inverted sterile petri dish and allow to sporulate for several hours (at least **02:00:00** and no more than **15:00:00**) onto the base of the dish.

-Replace the top of the dish, invert the dish and cover spores with room temperature Grace's + 5% FBS.

-Incubate dish for at least **48:00:00** at room temperature, monitoring for contamination. If the dish becomes uniformly cloudy at any point, it has been contaminated by something other than *E. muscae*.

-Once clumpy, protoplastic *E. muscae* growth can be observed, transfer culture to 25 cm^2 flask along with room temperature **Grace's + 5% FBS** to total final culture volume of **20 mL** .

1w

2 Incubate culture at room temperature until you reach late logarithmic growth. Culture morphology should still resemble heterogeneous small white clumps and lack obvious

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mycelial threads/tangles.

Harvest cells

3 Transfer at least **20 mL** of in vitro culture to sterile 50 ml conical tubes **Contributed by users** and spin 3500 rpm, **00:15:00** , 4C. This will produce a somewhat loose pellet. 15m

4 Decant most of supernatant, leaving loose pellet behind.

5 Resuspend the cells in any remaining supernatant (gently flick tube or pipette up and down with micropipettor) and transfer cell sludge to Axygen MaxyClear Tubes **VWR** **International Catalog #22234-046** 30s

6 Spin eppendorf tube for **00:05:00** at maximum rpm at **Room temperature** . Tight pellet will result. 5m

7 Aspirate supernatant and discard. 30s

8 Flash freeze tube contents using liquid nitrogen or dry ice + ethanol bath. 5m

8.1 If you are not ready to proceed with DNA extraction, cells should be stored at **-80 °C** .

DNA extraction - Day 1

9 **This DNA extraction is based on methods reported in Bulat et al, 1998** 5m

Bulat SA, Lübeck M, Mironenko N, Jensen DF, Lübeck PS. UP-PCR analysis and ITS1 ribotyping of strains of Trichoderma and Gliocladium.. Mycol Res.
http://10.1017/S0953756297005686

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10 Remove samples from cold bath or **-80 °C** and thaw at **Room temperature** .

11 In a fume hood, homogenize each sample with a Kontes Pellet Pestle 1.5 mL **VWR International Catalog #KT-749521-1590** then rinse pestle with **400 µL Buffer A**. 5m

12 Add **13 µL of proteinase K** (Proteinase K **NEB Catalog #P8107S** 20 mg/mL) to each sample to reach 600 ug/mL. 2m

12.1 This assumes pellet volume was approximately **20 µL** . If substantially deviated from this, adjust volume of proteinase K as needed to achieve desired concentration of 600 ug/mL.

13 Gently pipette suspension and mix further by gentle inversion, then briefly spin to collect all liquid in bottom of tube. 1m

14 Add **4.3 uL RNase A** RNase A **Fischer Scientific Catalog #FEREN0531** to each sample, mixing thoroughly by pipetting or gentle inversion, then briefly spin to collect all liquid in bottom of tube. 2m

15 Incubate **00:30:00** at **37 °C** . 30m

16 Store at **4 °C** **Overnight** . 30m

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DNA extraction - Day 2

17 Retrieve samples from fridge and put in chemical safety hood. Allow to come to **Room temperature** before continuing. 18h

18 Adjust salt concentration to 1 M NaCl of each sample by adding **104 µL** **4.4M NaCl**, mixing gently by inversion. 1m

19 Add **500 µL** **of 24:1 chloroform:octanol** to each sample and invert to mix. 2m

20 Incubate **00:15:00** at **Room temperature** . 15m

21 Spin **00:02:00** , 12,000xg, **Room temperature** 2m

22 Remove aqueous layer (i.e. top layer) and transfer to new tube. 5m

22.1 If volume in new tube less than **500 µL** , add **1 M NaCl** to bring up to **500 µL** . 1m

22.2 Sticky aqueous layer is a good sign!

23 Repeat steps 18) to 21) as necessary until you arrive with a non-cloudy aqueous layer. This may need to be repeated several times.

24 Spin out any residual protein via spin for **00:02:00** , 12,000xg, **Room temperature** . 2m

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25 Using a WIDE BORE TIP (cut P200 tip at first line with clean razor blade to generate wide opening) , transfer supernatant to new tube. 5m

26 Determine volume of supernatant using gradations on side of tube in combination with pipetting with wide-bore tip, as needed. 1m

27 Glycogen **Fisher** 2m

Add **3 µL of glycogen** (**Scientific Catalog #FERR0551**) to solution and mix gently by rocking back and forth.

28 Add **0.6 vol of isopropanol (IPA)** to supernatant. Invert GENTLY to mix and incubate at RT for **00:30:00** . 30m

29 Spin **00:10:00** ,max rpm, **Room temperature** to pellet out DNA. 10m

30 Wash pellet with **1 mL of 70% ethanol.** 1m

31 Spin **00:02:00** , max rpm, RT to re-pellet. 2m

32 Remove all supernatant with P1000/P200 and air-dry ~ **00:10:00** on kimwipe. 10m

33 WITH WIDE BORE TIP (cut P200 tip at first line with clean razor blade to generate wide opening) resuspend each pellet in desired volume TE buffer (**50 µL** - **100 µL**). 2m

Quality control - Perform same day as finishing extraction to minimize freeze-thaw cycles. Performing in this order should give most salient information first and save time if prep looks bad
35m

34 **Check polysaccharide and RNA contamination** 5m

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NanoDrop™ One UV-Vis Spectrophotometer
spectrophotometer

Thermo Scientific **ND-ONE-W**

Sample Volume (Metric): Minimum 1µL; Spectral Bandwidth: ≤1.8 nm (FWHM at Hg 254 nm); System Requirements: Windows™ 8.1 and 10, 64 bit; Voltage: 12 V (DC); Wavelength Range: 190–850 nm

1. Apply **1.5 µL** - **2 µL** of gDNA preparation to nanodrop and read A260/280 (RNA contamination); A260/230 (polysaccharide contamination).

34.1 We want to see:
- OD260/OD280 ratio between 1.8 and 2.0 (low protein contamination).
- OD260/OD230 ratio between 2.0 and 2.2 (low carbohydrate contamination).

Nanodrop will also give you **ballpark** for concentration to help select appropriate dilutions for Qubit in next step.

35 **Check DNA concentration** using Qubit™ dsDNA HS Assay kit **ThermoFisher Scientific Catalog #Q32851** 30m

Qubit 2.0 Fluorometer instrument **Q33226**
with Qubit RNA HS Assays

35.1 Prepare triplicate 1/10 and 1/100 dilution samples for Qubit per manufacturer protocol.

36 **Check size distribution**

This can be done via agarose gel or

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4200 TapeStation System
Electrophoresis tool for DNA and RNA sample quality control.

TapeStation Instruments **G2991AA**

36.1 Gel method:

1. Prepare 0.4% agarose gel in 1x TAE with NO intercalating agent.
2. Use the highest grade agarose you have access to!
3. Add back water after microwaving to maintain correct percentage of agarose.
4. Run out sample with high molecular weight fragment ladder (e.g. ⊠λ DNA-Mono Cut Mix **NEB Catalog #N3019S**) in 1x TAE at 3V/cm at RT until dye reaches ¾ way through gel (expect to take several hours (~4-5). Keep DNA in fridge/on ice during this time (DO NOT FREEZE).
5. Stain with ⊠SYBR™ Safe DNA Gel Stain **ThermoFisher Scientific Catalog #S33102** in water (1x solution) for 15 minutes at RT with gentle shaking.
6. Destain with water for 15 minutes at RT with gentle shaking.
7. Image with gel doc.

36.2 Tape Station method:

1. Bring ⊠Genomic DNA Reagents **Agilent Technologies Catalog #5067-5366** & ⊠Genomic DNA ScreenTape **Agilent Technologies Catalog #5067-5365** from 4C to RT for at least 30 min before starting.
2. Prepare 10-100 ng/uL dilution of your DNA.
3. Follow TapeStation Genomic DNA ScreenTape instructions to load samples and run.

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36.3 You could also use the

2100 Bioanalyzer Instrument
Sizing, quantification, and sample quality control of DNA, RNA, and proteins on a single platform
Agilent Technologies **G2939BA**

for this analysis, but it's a pain in the butt compared to the TapeStation.

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