**2023 UFIT Workshop: DNA extraction, purification, and shipping protocols**

You may proceed with one of the following options to extract your genomic DNA:

* Pure culture extraction protocol (Page 2)
* Plant tissue extraction protocol (Page 8) \*Note- This protocol has been successful when used with tomato plants in our lab, no guarantee with other plant material
* DNA extraction protocol of your choosing- commercial kits or other organic or nonorganic methods

*\*Extra purification steps are recommended if organic extraction methods (such as phenol-chloroform method) are used (Page 6 DNA purification)*

Important Note: DNA final amount must be between 5-10 μg (with concentration of 100 ng/μl or higher). DNA quality report from quantification must be sent to Dr. Pei-Ling Yu at plyu@ufl.edu by Thursday, January 12, 2023. Please see the last page of this protocol for report page to fill in and send. A separate file containing only the report page can also be found on our website. **Once your report is accepted and DNA is indicated as pure enough, it should be shipped to arrive by Wednesday, January 18. Please wait for confirmation of DNA quality/quantity via email before sending** **the sample.**

**Sample submission protocol:**

1. Make sure you receive the confirmation email from Dr. Yu. Print that email and include it and your DNA report with your sample.
2. Package your DNA sample in Parafilm-sealed or screw-top 1.5-mL microcentrifuge tubes and package in a cold shipping package (Styrofoam cooler with gel ice packs or dry ice will be sufficient). Ship overnight (afternoon arrival is fine) and indicate the email address below for notifications. Ship to arrive by Friday; the lab does not accept samples on weekends.

Ship your samples to:

UF-IFAS Plant Diagnostic Center

ATTN: Carrie Harmon, UFIT Workshop

2570 Hull Rd, Bldg. 1291

Gainesville, FL 32611-0830

+1 (352) 392-1795, PDC@IFAS.UFL.EDU

**DNA assessment methods**

We recommend the following methods for DNA quantification/qualification:

1. Nanodrop: concentration and purity
	1. 260/280 ~ 1.8
	2. 260/230 2.0~2.2
2. Qubit 1X dsDNA BR or HS assay kits (Q33265/ Q33230): concentration
3. Gel electrophoresis systems: DNA integrity
	1. For example, Agilent 2200 TapeStation Systems or gDNA gel electrophoresis
	2. DIN > 7, higher the better



To be considered as good DNA sample, it needs to meet the following criteria:

1. Concentration: >= 100 ng/μl
2. Quality:
	1. The ratio of absorbance at 260 nm and 280 nm: ~1.8
	2. The ratio of absorbance at 260 nm and 230 nm: 2.0~2.2
	3. High molecular weight gDNA: DIN > 7. To remove the degraded DNA fragments, we recommend you perform size selection using [SRE XS kit](https://www.pacb.com/wp-content/uploads/Insert-SRE-XS-kit.pdf) (SKU 102-208-200).
3. [Optional] If you have access to the Qubit fluorometer, the reading (concentration) should be close to the Nanodrop result. If your Nanodrop reading is much higher than the Qubit reading, you should consider purifying your DNA to remove contaminants.

Other quantification methods are welcome. Please fill out the form to report the readings of your DNA samples and send it to Pei-Ling Yu (plyu@ufl.edu) with subject “DNA sample quality report” by January 6th, 2023. Thank you.

[Read me]

Two protocols are provided here are for pure fungal culture and tomato leaves. The pure fungal culture extraction protocol has been tested on some Ascomycetes and Basidiomycetes. The plant tissue extraction protocol has been tested on tomato leaves so far. These two protocols contain two major steps: DNA extraction and DNA purification.

**Pure Fungal Culture Extraction**

**High-molecular weight genomic DNA**

Protocol adapted from Benjamin Schwessinger and Megan McDonald, High quality DNA from Fungi for Long Read Sequencing e.g., PacBio, Nanopore MinION Version 3, protocols.io

**Important notes before starting**

* Buffers are best when fresh and not older than 3-6 months. Buffered Phenol:Chloroform:Isoamylalcohol (25:24:1) should not be older than 3 months.
* Do NOT heat samples during DNA extractions! Perform all steps at RT or 4oC as indicated.
* Do NOT incubate samples with KAc for prolonged time periods
* Perform two steps of buffered Phenol:Chloroform:Isoamylalcohol purification to reduce co- purifying metabolites.

\*These notes are essential to obtain high quality DNA

**Reagents required:**

**BUFFER A:**

* 0.35 M sorbitol 0.1 M Tris-HCl, pH 9
* 5 mM EDTA, pH 8
* autoclave to sterilize

**BUFFER B:**

* 0.2 M Tris-HCl, pH 9 50 mM EDTA, pH 8
* 2 M NaCl
* 2% CTAB
* autoclave to sterilize

**BUFFER C:** 5% Sarkosyl (N-lauroylsarcosine sodium salt SIGMA L5125) Filter-sterilize

**Other solutions**:

* Potassium Acetate 5M (KAc precipitate polysaccharides) pH 7.5
* Polyvinylpyrrolidone (40000 MW) 10 % [w/v] (Sigma PVP40)
* Sodium Acetate (NaAc) 3M pH 5.2, Filter-sterilize
* Isopropanol 100%
* Ethanol 70%
* Buffered Phenol:Chloroforme:Isoamylalcool P:C:I (25:24:1, Sigma P2069)

**Enzymes**

* RNAse T1 (1000 U/mL, Thermo Fisher EN0541)
* Proteinase K (800U/mL, NEB P81072)

**Lysis Buffer For 17.5 mL for 500 mg starting material**

* 2.5 volume of Buffer A 6.5 mL
* 2.5 volume of Buffer B 6.5 mL
* 1.0 volume of Buffer C 2.75 mL
* PVP 10 % 1.75 ml

[**DNA purification kits**](https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/dna-purification/genomic-dna/qiagen-genomic-tips/?catno=10243)

QIAGEN Genomic-tip 100/G (Cat. No. / ID: 10243)

QIAGEN G2 buffer (Cat. No. / ID: 1014636)

QIAGEN Buffer QBT (750mM NaCl • 50 mM MOPS pH 7.0, 15% isopropanol (v/v), 0.15 % Triton X-100 (v/v)) (Cat. No. / ID: 19054)

QIAGEN Buffer QC (1M NaCl, 50 mM MOPs pH 7.0, 15% isopropanol (v/v)) (Cat. No. / ID: 19055)

QIAGEN Buffer QF (1.25 M NaCl, 50 mM Tris-Cl pH 8.5, 15% isopropanol (v/v)) (Cat. No. / ID: 19056)

**Protocol**

Extraction

**1.** Make lysis buffer by mixing buffer A+B+C (2.5:2.5:1 + 1%PVP final) and briefly heat to 64 °C. Let cool to room temperature for use in 50mL Falcon tubes.
All following steps are based on 17.5mL lysis buffer as starting volume.

**2.** Add 10 μl(10kU) RNAse A to lysis buffer

**3.** Grind tissue/spores with liquid nitrogen in a mortar with sand, use 1g of sand per 100mg of starting material. Grind for 2 mins in 4x 15 sec bursts adding liquid nitrogen after each 15 sec grinding burst.

**4.** Transfer powder to 50mL Falcon containing lysis buffer and RNAse, mix well by vortexing

**5.** Incubate at RT for 30 mins mixing by inversion every 5 mins

**6.** Add 200 μl Proteinase K, incubate at RT for 30 mins mixing by inversion every 5 mins

**7.** Cool on ice for 5 mins

**8.** Add 3.5 mL (0.2 vol) of KAc 5M, mix by inversion, incubate on ice for max 5 mins

**9.** Spin at 4°C and 5000g for 12 mins

**10.** Transfer supernatant to fresh Falcon tube containing 17.5mL (1vol) (P/C/I) and mix by inversion for 2 mins.

Supernatant may/may not have a lipid layer on the top (depends on the fungus), I don't really see a difference if I take this layer or not into the phenol/chloroform mix. I try to not take too much of it to make the next steps slightly easier.

**11.** Spin at 4°C and 4000g for 10 mins

**12.** Transfer supernatant (might be milky but do not worry) to fresh Falcon tube containing 17.5mL (1vol) P/C/I and mix by inversion for 2 mins

**13.** Spin at 4°C and 4000g for 10 mins

**14.** Transfer supernatant to fresh Falcon tube. If solution remains milky, repeat P/C/I wash. If solution is clear proceed to DNA precipitation.

DNA Precipitation

**15.** Add 1.8mL (0.1vol) NaAc and mix by inversion

**16.** Add 18mL (1vol) RT isopropanol and mix by inversion

**17.** Incubate at RT for 5-10mins or overnight at -20°C

**18.** Spin at 4 °C and 10000g for 30 mins

**19.** Carefully pour off supernatant until about 0.5 mL left, DNA will form a mostly translucent to white film/pellet at the bottom of the tube (color may vary depending on your fungus).

**20.** Use 1mL pipette tip to transfer pellet and remaining liquid into fresh 1.7mL Eppendorf tube.

* If DNA is not quite pure (as is the case for some fungal DNA extractions), pellet will be brittle and will break into small pieces. Try to recover as much as possible with 1mL pipette.
If the pellet breaks apart too much, add 1.5mL fresh 70% EtOH to the 50mL Falcon and spin for 5 min at 4000g.
Remove 1mL and transfer the remaining volume and DNA pellet to same 2mL Eppendorf tube.

**21.** Spin in tabletop centrifuge for 5 mins at 13000g

**22.** Remove supernatant with pipette and wash with 1.5mL fresh 70% ethanol, invert several times to dislodge pellet

**23.** Spin in tabletop centrifuge for 5 mins at 13000g. Repeat wash steps (21-21).

**24.** Pour off ethanol and remove remaining ethanol with pipette. Spin down briefly and remove any remaining ethanol with pipette.

**25.** Air-dry pellet for 5 mins

**DNA Purification**

26. Add 4 mL of Qiagen G2 buffer and dissolve the pellet at 65°C for 20 min if necessary. Mix by inversion, don’t vortex.

27. Centrifuge the sample at 5,500 g at 4°C for 5 minutes, then transfer the supernatant to the new tube.

28. Pre-warm Buffer QF to 55°C.

29. Equilibrate a Qiagen Genomic-tip 100/G column with 4 mL of Buffer QBT.

30. Apply the re-suspended DNA to the column.

31. Wash the column twice with 7.5 mL of Buffer QC.

32. Elute the DNA with 5 mL of pre-warmed Buffer QF.

33. Precipitate the DNA using 3.5 mL of isopropanol at RT.

34. Centrifuge at 5,500 g for 30 min. at 4°C.

35. Wash the pellet in 2mL of cold 70% ethanol. Dry the pellet and re-suspend in ~50-100 μl water.

36. Assess the DNA using Nanodrop, gel electrophoresis, Qubit and/or tapestation.

If necessary, use the Short Read Eliminator kit and re-assess the DNA.

**Plant Tissue Extraction Protocol**

* DNA extraction buffer

|  |  |  |  |
| --- | --- | --- | --- |
| Chemical | Final concentration | Stock / 100 mL | Stock preparation |
| Sorbitol | 350 mM | 17.5 mL - 1 M | 2 M = 364.34 g/l |
| Tris-Cl | 100 mM | 10 mL - 1 M | 1 M = 121 g/l |
| EDTA | 5 mM | 1 mL - 0.5 M | 0.5M = 186 g/l |
| DI Water |  | 71.5 mL |  |

* Nuclei lysis buffer:

|  |  |  |  |
| --- | --- | --- | --- |
| Chemical | Final concentration | Stock / 100 mL | Stock preparation |
| Tris-Cl | 200 mM | 20 mL - 1 M | 1 M = 121 g/l |
| EDTA | 50 mM | 10 mL - 0.5 M | 0.5M = 186 g/l |
| NaCl | 2 M | 40 mL - 5 M | 5 M = 292.2 g/l |
| CTAB\* | 2% | 20 mL - 10% | 10% = 100 g/l |
| DI Water |  | 10 mL |  |

* Sarkosyl\* 5%:

|  |  |  |
| --- | --- | --- |
| Chemical | Final concentration | Stock |
| Sarkosyl\* | 5% w/v | 5 g |
| DI Water |  | Up to 100 mL |

* Micro-prep buffer:



* Sodium bisulfate

Freshly add to micro-prep buffer before use.

0.05 g of to 10 mL of micro-prep buffer

* Chloroform: Isoamyl alcohol(24:1)
* Isopropanol
* Ethanol (70%)
* TE buffer (pH 8.0) w/ RNAase (20 µg/mL) (**freshly prepared**)

|  |  |  |
| --- | --- | --- |
| Chemical | Concentration | Per 500 µl |
| RNAase | 20 µg/mL | 1 µl |
| TE Buffer |  | 500 µl |

* Add after autoclaving

# DNA extraction

# Flash freeze in liquid nitrogen, lyophilize, and store in -80 °C if necessary.

# ***Freshly prepare micro-prep buffer*** as mentioned above and keep at room temperature.

# Add 50 to 100 mg of ground tissue in 10 mL of buffer and add 200 μl of RNAse (stock 10 mg/mL) in each tube.

# (***If you are using fresh young leaf tissues, start materials need to be increased to 500 mg to 1 g***)

# Shake gently by hand. DO NOT VORTEX.

# Incubate in 65°C water bath for 2 hours. Invert tube multiple times every 20 min.

# Add 10 mL of chloroform:isoamyl (24:1) to each tube.

# Mix well by inverting. DO NOT VORTEX.

# Centrifuge at 5,500 rpm for 10 minutes.

# Pipette off aqueous phase (about 9 mL) into new microcentrifuge tube.

# Reap 1 part of chloroform:isoamyl (24:1) to each tube

# Repeat step 8 to 10.

# Add 3 parts cold isopropanol to 5 parts aqueous phase [4.8 mL of isopropanol + 8 mL of aqueous phase]

# Invert tubes repeatedly until DNA precipitates out.

# Immediately centrifuge at 5,500 rpm for 30 minutes.

# Pour off supernatant. DO NOT DISTURB THE PELLET.

# Add 750 μl ice cold 70% Ethanol. (Can be stored in -20°C indefinitely)

# Centrifuge at 5,500 rpm for 5 minutes.

# Pour off supernatant and dry pellet by air dry for 5 minutes.

# Proceed to Purification steps or step 20.

*Purification is highly recommended for cleaner sample*

# Resuspend in 50 ~ 100 μl water at 65°C for 15 min.

# Spin the tube for 5 min at 5,500 rpm.

# Quantify the DNA concentration with Nanodrop, Qubit, and/or TapeStation.

# Notes:

# For young leaves, you may yield 0.2 ug of DNA per mg of the tissue. The yield drops if older tissues are used.

# Purification

1. Add 4 mL of Qiagen G2 buffer and dissolve the pellet at 65°C for 20 min if necessary. Mix by inversion, don’t vortex.
2. Centrifuge the sample at 5,500 g at 4°C for 5 minutes, then transfer the supernatant to the new tube.
3. Pre-warm Buffer QF to 55oC.
4. Equilibrate a Qiagen Genomic-tip 100/G column with 4 mL of Buffer QBT.
5. Apply the re-suspended DNA to the column.
6. Wash the column twice with 7.5 mL of Buffer QC.
7. Elute the DNA with 5 mL of pre-warmed Buffer QF.
8. Precipitate the DNA using 3.5 mL of isopropanol at RT.
9. Centrifuge at 5,500 g for 30 min. at 4 oC.
10. Wash the pellet in 2mL of cold 70% ethanol. Dry the pellet and re-suspend in ~50-100 μl water.
11. Assess the DNA using Nanodrop, gel electrophoresis, Qubit and/or TapeStation.
12. If necessary, use the Short Read Eliminator kit and re-assess the DNA.

**DNA sample quality report**

|  |  |
| --- | --- |
| **Name** |  |
| **Organization** |  |
| **Contact information** | Email:  |
| **DNA extraction method(s)** |  |
| **Host** |  |
| **Potential fungal pathogen** | *(N/A if unknown)* |
| **DNA concentration**  | Method: Nanodrop or other\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_ ng/ul |
| Method: Qubit 1X DNA BR Assay kit \_\_\_\_\_\_ ng/ul |
| Method:  |
| **Gel image** |  |
| **DNA Integrity Number****(If any)** |  |
| **DNA purity** | 260/280 = \_\_\_\_\_\_\_\_  |
| 260/230 = \_\_\_\_\_\_\_\_ |
| **Notes** |  |