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Enrichment and Extraction of Phytoplasma DNA from Field Samples for Whole-Genome Sequencing

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We use this protocol and it's working

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Abstract

This protocol enables the direct enrichment of phytoplasma DNA from field samples, eliminating the need for propagation in test plants. It was developed using grapevine samples with low Flavescence dorée phytoplasma concentrations and incorporates both pre- and post-extraction enrichment steps to enhance the suitability of the extracted DNA for high-throughput sequencing (HTS) aimed at whole-genome analysis. Whole-genome amplification is an optional final step but is required when sequencing with Oxford Nanopore Technologies platforms.

Guidelines

1) This protocol is optimized for phytoplasma-infected grapevine samples with low phytoplasma concentrations.
3) Work in a clean environment and use dedicated equipment to prevent cross-contamination between samples.
4) Ensure all reagents are prepared fresh or stored according to manufacturer recommendations.
5) When using Oxford Nanopore Technologies (ONT) sequencing, whole-genome amplification is required to obtain sufficient DNA input.
6) While this protocol was developed for grapevine samples, it may be adapted for other naturally infected plant hosts. The effectiveness of enrichment steps may vary depending on phytoplasma titer and sample quality. Optimization may be necessary for different host species or environmental conditions.
7) Use a fluorometer or qPCR to quantify DNA yield after extraction and enrichment.

Materials

liquid nitrogen
K_2HPO_4
KH_2PO_4
saharose
bovine serum albumin (BSA)
polyvinylpyrrolidone-10 (PVP10)
ascorbic acid
CTAB
polyvinylpyrrolidone-40 (PVP40)
Tris-HCl
NaCl
EDTA
chloroform:isoamyl alcohol (24:1)
isopropanol
70% ethanol
nuclease free water
NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs, Massachusetts, USA, cat no. E2612S)
REPLIg-midi Kit (Qiagen, Hilden, Germany, cat no. 150043)

Safety warnings

1) If working with quarantine phytoplasmas, follow appropriate biosafety regulations and disposal procedures.
2) The sample preparation has to keep homogenized material frozen to minimize DNA degradation.
3) The CTAB extraction method involves hazardous reagents (e.g., chloroform). Perform extractions in a fume hood, wear gloves, and dispose of chemical waste according to institutional safety guidelines.

Before you start

1 Prepare PGB (phytoplasma grinding buffer) pH 7.6

	Chemical	Final concentration
	K2HPO4	0.096 M
	KH2PO4	0.03 M
	saharose	10 %
	BSA	0.15 %
	PVP10	2 %
	ascorbic acid*	0.03 M

BSA - bovine serum albumin
PVP10 - polyvinylpyrrolidone-10

***Add ascorbic acid just before adding PGB to sample!**

The initial buffer without ascorbic acid can be stored up to one month at 4°C.

2 Prepare CTAB buffer pH 8

	Chemical	Final concentration
	CTAB	4 %
	PVP40	1 %
	Tris-HCl	0.2 M
	NaCl	1.4 M
	EDTA	0.02 M

Store the buffer at room temperature.

3 Store aliquot of isopropanol and 70% ethanol to -20°C and warm up CTAB buffer to 65°C.

Plant material preparation and homogenisation

4 Cut out leaf veins from fresh leaves taken from different parts of the plant.

5 Chop leaf veins into small pieces with sterile scalpel.

6 Homogenize chopped leaf veins with liquid nitrogen with mortar and pestle.

7 Use a pre-cooled spatula and transfer 1 g of the powdered plant tissue sample into a pre-cooled 15 mL tubes.

Warning! Keep the material frozen at all times!
Option: store homogenized material at -80°C until further analysis.

Pre-extraction phytoplasma enrichement: differential centrifugation

8 To 1 g of frozen homogenized sample add 10 mL of PGB and put on ice.

9 Keep on ice for 10 min.

10 Vortex (watch out that the centrifuge does not get warm) and short spin. 0 rpm

11 Pour the extract into pre-cooled 15 mL tubes and add PGB buffer for balancing with other samples for centrifugation.

12 Centrifuge 5 min at 2500 g and 4°C.

13 Pour the supernatant to fresh pre-cooled 15 mL tubes (**Warning!** These tubes need to withstand high speed centrifugation). Balance them carefully using scale and PGB.

14 Centrifuge 25 min at 18,000 g and 4°C.

15 Carefully pour out the supernatant and drain the tube on paper towel. Keep the pellet.

Total DNA extraction

16 Add 2 ml of 4% CTAB buffer (warmed up to 65°C) to the pellet after differential centrifugation and resuspend by vortex.

17 Incubate at 65°C for 1 hour (vortex in between).

18 Centrifugate: 5 min, 5000 g, room temperature.

19 Divide one sample into 2 parallels for easier manipulation: transfer two times 1 ml of the sample to a 2 ml tube and add the same amount of chloroform:isoamyl alcohol (24:1) to each tube and vortex.

20 Centrifugate: 10 min, 6000 g, room temperature.

21 Transfer the upper aqueous phases to a fresh 1.5-tubes and add an equal amount of ice-cold isopropanol to each tube and mix by inverting.

22 Precipitate DNA for 20 min at room temperature.

23 Centrifugate: 15 min, 15,000 g, 4°C.

24 Wash the pellets with 300 µl ice-cold 70% ethanol.

25 Centrifugate: 2 min, 10,000 g, 4°C.

26 Pipet out the ethanol and dry the pellet.

27 Resuspend the pellet in 50 µl of nuclease free water.

28 Combine parallel extractions in one tube.

Post-extraction phytoplasma enrichment

29 Follow manufacturers protocol for the NEBNext® Microbiome DNA Enrichment Kit (New England Biolabs,
Massachusetts, USA) using 80 µl of the extracted DNA.

Optional: whole genome amplification

30 Use the REPLIg-midi Kit (Qiagen, Hilden, Germany) with an input of 10 ng of enriched DNA and follow Protocol for Amplification of Purified Genomic DNA using the REPLI-g Midi Kit.

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